The Effects of Hypoxia on Human Adipose Tissue Lipid Storage and Mobilization Functions: From Primary Cell Culture to Healthy Men

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THESIS ABSTRACT

Adipose tissue plays a central role in the regulation of lipid storage and mobilization. A tight control between adipose tissue lipid storage and mobilization functions must be exerted to prevent an overload of lipids at other organs such as the heart, liver and skeletal muscles, and favor the risk of developing metabolic disorders, such as Type 2 diabetes and cardiovascular diseases (CVD). There is strong evidence from animal studies that low oxygen levels (hypoxia) are noted in adipose tissue as the mass of the organ excessively expands and, in turn, exacerbates some adipose tissue functions. Whether hypoxia exposure, which could be derived from reduced environmental oxygen availability, disease or a combination of both, affects adipose tissue lipid storage and mobilization functions in humans is not well known. Using *in vitro* and *in vivo* approaches, this thesis aimed at characterizing the effects of hypoxia on human adipose tissue lipid storage and lipid mobilization functions. Study I investigated how hypoxia can modulate human adipose functions such as lipid storage and lipid mobilization *in vitro*. Study II examined whether acute intermittent hypoxia, which simulates obstructive sleep apnea, affects adipose tissue lipid storage/mobilization functions and triglyceride levels in healthy young men in postprandial state. Study III tested the effect of an acute 6-hour continuous exposure to hypoxia (fraction of inspired oxygen (FIO_2) = 0.12)) on plasma triglyceride levels in healthy young men in the fasting state. Study I indicates that both acute (24h) and chronic (14d) hypoxia (3%, and 10% O_2) modulate human adipose tissue lipid storage and mobilization functions in a different manner. Study II demonstrates that acute exposure to intermittent hypoxia (6h) is sufficient to increase plasma non-esterified fatty acids (NEFA) levels, as well as insulin levels, but does not alter circulating triglyceride or subcutaneous adipose tissue lipid storage and/or mobilization

capacity *ex vivo* in healthy men. Study III shows that acute exposure to normobaric hypoxia increases circulating NEFA and glycerol concentrations but did not translate in altering circulating triglycerides in fasting healthy men. In conclusion, our observations suggest that an exposure to reduced oxygen levels impairs human adipose tissue storage and/or mobilization functions, a phenomenon known in the development of metabolic disorders, such as Type 2 diabetes and CVD.

RÈSUMÈ DE THÈSE

Le tissu adipeux joue un rôle central dans la régulation de l'entreposage et la mobilisation des lipides. Un contrôle précis entre ces fonctions se doit d'être exercé pour prévenir une surcharge de lipides aux autres organes tels le cœur, le foie et les muscles squelettiques puisque ceci favorise le risque de développement de désordres métaboliques comme le diabète de type 2 et les maladies cardiovasculaires (MCV). Des évidences claires issues d'études réalisées chez l'animal suggèrent que de faibles niveaux d'oxygène (hypoxie) sont notés dans le tissu adipeux en fonction de l'expansion de la masse de ce dernier, conduisant ainsi à des altérations des fonctions du tissu adipeux. Il est encore peu connu si l'exposition à l'hypoxie, qu'elle soit dérivée d'une exposition à un environnement réduit en oxygène, d'une maladie ou une combinaison des deux, affecte les fonctions d'entreposage et de mobilisation des lipides du tissu adipeux. À l'aide d'approches *in vitro* et *in vivo*, cette thèse vise à caractériser les effets de l'hypoxie au niveau des fonctions d'entreposage et de mobilisation des lipides du tissu adipeux. L'étude 1 documente comment l'hypoxie module les fonctions d'entreposage et de mobilisation des lipides du tissu adipeux *in vitro*. L'étude II examine si l'hypoxie intermittente, qui simule l'apnée obstructive du sommeil, affecte les fonctions d'entreposage et de mobilisation des lipides du tissu adipeux et les triglycérides circulants de jeunes hommes en santé en situation postprandiale. L'étude III teste les effets D'une exposition aiguë de 6 heures d'hypoxie continue (fraction d'oxygène inspirée (FIO_2) = 0.12) chez des jeunes hommes en santé à jeun. L'étude 1 indique qu'une exposition à l'hypoxie aiguë (24h) ou chronique (14 jours) (3% et 10% O_2) module les fonctions d'entreposage et de mobilisation des lipides du tissu adipeux de façon différente. L'étude II démontre qu'une exposition aiguë à l'hypoxie intermittente (6h) est suffisante pour augmenter

les concentrations plasmatiques d'acides gras nonestérifiés (NEFA), les concentrations d'insuline, sans toutefois altérer les triglycérides circulants ou la capacité d'entreposage et de mobilisation des lipides du tissu adipeux ex vivo de jeunes hommes en santé. L'étude III montre qu'une exposition aiguë à l'hypoxie normobarique augmente les acides gras nonestérifiés et le glycérol circulants sans altérer les concentrations de triglycérides de jeunes hommes en situation de jeune. En conclusion, nos observations suggèrent qu'une exposition à des niveaux réduits d'oxygène détériore les fonctions d'entreposage et de mobilisation des lipides du tissu adipeux, un phénomène reconnu dans le développement de désordres métaboliques comme le diabète de type 2 et les MCV.

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First, I would like to thank my supervisor, Dr. Pascal Imbeault, for his guidance and support over the last 4 years throughout my degree. The support and training he provided was beyond expectation, and this has helped me look forward to my next career steps with great confidence. I would also like to thank Drs. Eric Doucet and Glenn Kenny for serving as thesis supervisory committee members, and providing important feedback on the proposed projects.

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I must also acknowledge the various agencies that provided scholarship support throughout my PhD Program. The funds were provided by the Faculty of Graduate and Postdoctoral Studies (FGPS), and the Ontario Graduate Scholarship (OGS). Travel bursaries were provided by the FGPS and Graduate Students Association of the University of Ottawa (GSAED) for me to attend and present at various scientific conferences.

Finally, I must express my gratitude to the volunteers who graciously participated in my thesis studies. The numerous visits, blood samples and fat biopsies collected and hypoxia exposures were challenging and uncomfortable for them, so I thank them for their effort and co-operation.

LIST OF STUDIES

STUDY I

Mahat, B., Mauger, J.-F., and Imbeault, P. Effects of Different Oxygen Tensions on Differentiated Human Preadipocytes Lipid Storage and Mobilization Functions. *In progress of manuscript writing.*

STUDY II

Mahat, B., Chassé, É., Mauger, J.-F., and Imbeault, P. 2016. Effects of acute hypoxia on human adipose tissue lipoprotein lipase activity and lipolysis. J. Transl. Med. **14**(1): 212. doi:10.1186/s12967-016-0965-y.

STUDY III

Mahat, B., Chassé, É., Clare L, Mauger, J.-F., and Imbeault, P. No Effect of Acute Normobaric Hypoxia on Plasma Triglyceride Levels in Fasting Healthy Men. Manuscript in revision to *Applied Physiology, Nutrition, and Metabolism*.

PREFACE

The work presented herein is my own, and I take full responsibility for its contents. All thesis studies in Chapter 3 were co-authored by Drs. Pascal Imbeault and Jean-Francois Mauger. Additionally, Study II and III was co-authored by Etienne Chasse, and Study III was co-authored by Clare Lindon. At the time of thesis submission, the data collection of Study I was just finished, so Study I is in progress of manuscript writing. The partial data of Study I are also present in Study II, which are included in Chapter 3. Study II was published in *Journal of Translational Medicine*. Furthermore, Study III is under revision to *Applied Physiology, Nutrition, and Metabolism*. Ethical approval from the University of Ottawa was required for Study II and III studies, which are included in Appendix A. The published versions of Study II can be found in Appendix B.

In addition to the thesis studies in Chapter 3, a list of published abstracts during my PhD tenure can be found in Appendix C. Permission for republication of Study II article in a thesis was not required, since the *Journal of Translational Medicine* is under the terms of BioMed Central Open Access (see Appendix D). Figure 2 presented in Chapter 2 was reasonably modified, so republication permission was not acquired. However, the reference of Figure 2 presented in Chapter 2, was properly addressed. Finally, republication permissions of Table 1 presented in Chapter 2, published in *Physiological Review* was not required, and these can be found in Appendix D.

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THESIS DISCUSSION

LIST OF ABBREVIATIONS

Term	Description
O_2	Oxygen
N_2	Nitrogen
ATP	Adenosine triphosphate
OSA	Obstructive sleep apnea
COPD	Chronic obstructive pulmonary disease
CVD	Cardiovascular disease
TG	Triglycerides
NEFA	Non-esterified fatty acids
LPL	Lipoprotein lipase activity
MGAT	Monoacylglycerol acyltransferase activity
DGAT	Diacylglycerol acyltransferase activity
ATGL	Adipose triglyceride lipase
HSL	Hormone-sensitive lipase
VLDL	Very-low density lipoprotein
LDL	Low-density lipoproteins
ApoB	Apolipoprotein B
DNL	*De novo* lipogenesis
FAS	Fatty acid synthase
ACC	Acetyl-coA carboxylase
SREBP1	Sterol regulatory element binding protein 1
ChREBP	Carbohydrate response element-binding protein
ANGPTL-4	Angiopoietin like-4 protein

DGs	Diglycerides
MGs	Monoglycerides
SNS	Sympathetic nervous system
AR	Adrenoceptors
cAMP	Cyclic adenosine monophosphate
PKA	Protein kinase A
PDE	Phosphodiesterase
PIK	Phosphatidyl inositol kinase
PO_2	Partial pressure of oxygen
WAT	White adipose tissue
RDI	Respiratory disturbance index
BMI	Body mass index
PKB	Protein kinase B
HIF-1	Hypoxia-inducible factor 1
TNF-α	Tumor necrosis factor alpha
CHO	Carbohydrate
IL-6	Interleukin 6
FiO_2	Fraction of inspired oxygen
HR	Heart rate
BPM	Beats per minute
VO_2 max	Maximum volume of oxygen
SpO_2	Oxyhemoglobin saturation
NaCl	Sodium chloride
HL	Hepatic triglyceride lipase
AMPK	Adenosine monophosphate-activated protein kinase

LIST OF DEFINITIONS

1) Hypoxia: It is a condition in which the body or region of the body is deprived of adequate oxygen at the tissue level. It is created either by environmental conditions like high altitude exposure, or by pathological conditions such as chronic obstructive pulmonary disease, obstructive sleep apnea (OSA) or severe anemia (Deldicque and Francaux 2013).

2) Chronic hypoxia: The term "chronic hypoxia" is used for hypoxia conditions lasting for several days (Deldicque and Francaux 2013) for instance, Young et al. (Young et al. 1987) considered chronic hypoxia as hypoxia lasting for 13 days.

3) Acute hypoxia: The term "acute hypoxia" is used for hypoxia conditions for a period of several hours (Deldicque and Francaux 2013), for instance, Young et al. (Young et al. 1987) considered acute hypoxia as hypoxia for period of less or equal to 24 hours.

4) Intermittent hypoxia: It is broadly defined as repeated episodes of hypoxia interspersed with episodes of normoxia (Neubauer 2001).

5) Hypoxemia: It is simply a decrease in oxygen saturation of hemoglobin which may lead to hypoxia in tissues.

6) Obesity: It is the result of an imbalance between energy intake and energy expenditure. When the energy intake exceeds the energy expenditure, there is an energy surplus that is stored mainly in adipose tissue (Landini et al. 2016).

7) Obstructive sleep apnea: It is a highly prevalent disorder characterized by repetitive upper airway obstruction during sleep that leads to intermittent hypoxia, sleep fragmentation and excessive daytime sleepiness (Garvey et al. 2009).

8) Chronic obstructive pulmonary disease: It is the group of lung diseases that includes chronic bronchitis, emphysema, in some cases chronic asthma, and others, which are characterized by restricted airflow. When the supply of oxygen to the lungs is restricted or limited, it increases the risk of chronic obstructive pulmonary disease patients to have hypoxia.

9) High altitude: Altitude is defined by the vertical distance to sea level. A high altitude is usually ≥3,000 m (Bärtsch et al. 2008), which can lead to a decrease in the oxygen content of the human body.

10) Lipid: It is a basic term representing a molecule that is fat soluble (vs water soluble). Fatty acids, sterols and triglycerides (TG) all fall under the category of lipid.

11) Triglycerides: Most energy reserves in the human body are stored as TG and composed of a glycerol and three fatty acids (Frayn et al. 2006).

12) Fatty acids: They are usually derived from TG. They are important dietary sources of fuel for animals because, when metabolized, they yield large quantities of adenosine triphosphate (ATP).

13) Non-esterified fatty acids: Any fatty acid which occurs free, rather than esterified with glycerol to form a glyceride or other lipid, usually as the result of hydrolysis.

14) Chylomicrons: They are lipoprotein particles consists of TG, phospholipids, cholesterol, and protein. They transport dietary lipids from the intestines to other locations in the body (Hussain 2000).

15) Very-low density lipoprotein: It is a type of lipoprotein made by the liver. It is assembled in the liver from TG, cholesterol, and apolipoproteins (Gibbons et al. 2004).

16) Adipose tissue: It is the body's largest energy organ with more than 95% of the body's lipids, stored as TG (Coppack et al. 1994). It plays a central role in energy substrate homeostasis by acting as a crucial regulator of whole-body lipid flux.

17) Lipoprotein lipase: It is the rate-limiting enzyme for the hydrolysis of the TG core of circulating TG-rich lipoproteins, chylomicrons, and very low-density lipoproteins (VLDL). It degrades circulating TG to fatty acids for their subsequent uptake within the adipose tissue where they can be synthesized into TG (Kersten 2001, Shi and Cheng 2009).

18) Lipogenesis: The excess of energy is stored in the form of TG, a process termed lipogenesis (Björntorp 1996, Frühbeck et al. 2001) that is partly driven by the enzymatic action of lipoprotein lipase (Luo and Liu 2016).

19) Lipolysis: In the time of increased metabolic need, lipid storage can be mobilized by converting adipose tissue TG into fatty acids, a process called lipolysis ("Lipolysis, Fat Mobilization, Fatty Acid (beta, alpha, omega) Oxidation, Ketogenesis" n.d., Lass et al. 2011).

20) *De novo* lipogenesis: The synthesis of new fatty acids, mainly during the postprandial state, is considered as *de novo* lipogenesis ("Adipose tissue de novo lipogenesis" n.d., Letexier et al. 2003).

CHAPTER 1: INTRODUCTION

Oxygen (O_2) is known to have a major role in vegetal and animal respiration (Brahimi-Horn and Pouysségur 2007). At the cellular level, mitochondria utilizes O_2 to produce adenosine triphosphate (ATP) via the biochemical process of oxidative phosphorylation (Semenza 2000, Kumar 2016). A dysfunction of oxidative phosphorylation leads to severe conditions or even death. Therefore, humans have a highly regulated mechanism to sense small fluctuations of O_2 tension in tissues. Certain circumstances can cause a restriction in O_2 supply and/or increased O_2 consumption, which may lead to oxyhemoglobin desaturation and tissular hypoxia (Brahimi-Horn and Pouysségur 2007, Johnson et al. 2010). One of the circumstances may occur in individuals with obesity, where there is an ongoing debate on whether excessive adipose hypertrophy, can impair O_2 diffusion in the adipose tissue and lead to adipose tissue hypoxia in humans (Pasarica et al. 2009, Goossens et al. 2011, Trayhurn and Alomar 2015). Other circumstances that affects the supply of O_2 are obstructive sleep apnea (OSA) (Young et al. 2002), chronic obstructive pulmonary disease (COPD) (Raguso et al. 2004, Baldi et al. 2010), and exposure to high altitudes (Surks et al. 1966, Leaf and Kleinman 1996). Individuals with OSA experience short periods of hypopnea, inducing intermittent hypoxia-hypercapnia/normoxia cycles. Intermittent hypoxia induces a temporary hypoxemia that can go as low as 60% during OSA (Government of Canada 2010). Hypoxemia is simply a decrease in O_2 saturation of hemoglobin which may lead to hypoxia in tissues. Additionally, individuals with COPD show chronic hypoxemia which may also affect adipose tissue function (van den Borst et al. 2013). Finally, decrease in O_2 content of the human body can also occur during exposure to high altitude

(≥3,000 m) (Surks et al. 1966, Young et al. 1989, Bärtsch et al. 2008). In 1998, there were 135,00000 people living above 3500m which represented about 0.002% of the world population (Cohen and Small 1998).

Important health consequences of individuals with obesity (Blüher 2009, McQuaid et al. 2011), OSA (Drager et al. 2010, Government of Canada 2010), COPD (Cebron Lipovec et al. 2016), and exposed to high altitudes conditions (Siqués et al. 2007) are an increased risk of developing metabolic disorders such as Type 2 diabetes and cardiovascular disease (CVD). A potential explanation underlying obesity, OSA, COPD, and high altitude exposure with increased risk of metabolic disorders resides on the possibility that obesity, OSA, COPD, and high altitude conditions may disturb lipid storage and mobilization functions, thereby leading to a deteriorated blood lipid profile. More precisely, this altered lipid profile is featured by an increase in triglyceride (TG) levels. It has been shown that individuals living with obesity (Tiihonen et al. 2015, Khan and Khaleel 2016) display increased TG (by ~60%), individuals with OSA (Newman et al. 2005) showed increased TG (by ~30%), individuals with COPD display either increases (by~30%) (Mitra et al. 2015, Ameen et al. 2016), decreases (by~20%) (Sin and Man 2003), or no change (Fekete and Mösler 1987, Basili et al. 1999), in TG, and individuals at high altitudes showed increases (+44% (Whitten and Janoski 1969), +81% (Young et al. 1989), +47% (Siqués et al. 2007)), decreases (-42% (Férézou et al. 1988), -19% (Stöwhas et al. 2013)) or no change (Leaf and Kleinman 1996) in plasmatic TG, compared to individuals without obesity, OSA, COPD, and not exposed to high altitudes.

Adipose tissue plays a central role in energy substrate homeostasis by acting as a crucial regulator of lipid storage and mobilization (Luo and Liu 2016). More specifically, the excess of energy is stored in the form of TG, a process termed lipogenesis that is partly driven by the

lipoprotein lipase (LPL) (Luo and Liu 2016). LPL degrades lipoprotein-bound TG to fatty acids for their subsequent uptake by the adipose tissue where they can be re-esterified into TG (Kersten 2001, Shi and Cheng 2009). Conversely, in time of increased metabolic need, stored lipid can be mobilized by converting adipose tissue TG into fatty acids using a process called lipolysis ("Lipolysis, Fat Mobilization, Fatty Acid (beta, alpha, omega) Oxidation, Ketogenesis" n.d., Lass et al. 2011). Fatty acids derived from lipolysis are released into circulation and delivered to peripheral tissues for sustaining energy demand.

A tight control between adipose tissue lipid storage and mobilization functions must be exerted. Impaired lipid storage and/or excessive mobilization of lipid stores can overload other organs such as the heart, liver, and skeletal muscles with lipids, which refers to stimulate ectopic fat deposition. This 'lipotoxic' phenomenon is well recognized to precede the development of metabolic disorders such as CVD (DeFronzo 2004, Lelliott and Vidal-Puig 2004, Slawik and Vidal-Puig 2006). A better appreciation of how hypoxia affects human adipose tissue storage and mobilization functions could facilitate the treatment and prevention of metabolic disorders such as Type 2 diabetes and CVD.

1.1 Rationale and statement of the problem

First, a paucity of *in vitro* studies tried to determine the effects of hypoxia on human preadipocytes lipid storage and mobilization functions (Famulla et al. 2012, O'Rourke et al. 2013). These previous studies provide evidence that hypoxia alter the metabolism of human adipocytes *in vitro*. However, these studies differed in terms of *in vitro* modalities of exposure to hypoxia. Consequently, these previous *in vitro* observations regarding the effects of hypoxia on

human preadipocytes lipid storage and mobilization function need to be further consolidated and validated.

Second, recent evidence from animal studies suggest that O_2 deprivation, can substantially raise plasma TG concentrations and delay blood lipid clearance (Muratsubaki et al. 2003, Drager et al. 2012, Jun et al. 2012, 2013, Yao et al. 2013). These changes appear to be caused, in part, by a) an increase in lipid influx to the liver due to an increase in adipose tissue mobilization of lipid and b) the suppression of the adipose tissue storing capacity activity (Jun et al. 2012). Animal studies also suggest that TG response to hypoxia may be related to the nutritional status by increasing plasma TG levels in postprandial state and no increase in plasma TG levels in fasting state when exposed to hypoxia (Muratsubaki et al. 2003). However, it is still unknown whether these observations obtained *in vitro* and in animal models regarding the effects of hypoxia on adipose tissue functions as well as on postprandial and fasting TG concentrations also occur *in vivo* in humans.

The studies outlined below are designed to answer the above queries by investigating the *in vitro* and *in vivo* effects of hypoxia on human adipose tissue lipid storage and mobilization functions.

1.2 Objectives

The proposed thesis aims to answer the following questions:

1) How hypoxia affects the lipid storage and mobilization functions on differentiated human preadipocytes?

2) Does acute intermittent hypoxia affect plasma TG and adipose tissue lipid storage and mobilization functions in healthy men in postprandial state?

3) Does an acute continuous exposure to hypoxia affect plasma TG levels in fasting healthy humans?

1.3 Hypotheses

Our hypotheses were:

1) Hypoxia would inhibit LPL activity and reduce the expression of genes involved in lipid storage as well as stimulate the lipolytic activity of differentiated human preadipocytes.

2) Acute intermittent hypoxia would lead to an exaggerated elevation in postprandial TG concentrations consequent to an increase in adipocyte lipolysis and/or impairment in subcutaneous abdominal adipose tissue LPL activity in healthy men.

3) Acute exposure to continuous hypoxia in the fasting state (low insulinemia) would increase circulating NEFA concentrations and TG levels in healthy men.

1.4 Implications

Hypoxia is well recognized to induce many rescue pathways including augmenting glycolytic flux and reducing oxidative glucose oxidation, mainly catalyzed by changes orchestrated by the transcription factor hypoxia inducible factor-1 (HIF-1) (Semenza 2014, 2017). Less emphasis has been given on the impact of hypoxia on lipid mobilization and storage functions, key determinants in the development of metabolic disorders (DeFronzo 2004, Lelliott and Vidal-Puig 2004, Slawik and Vidal-Puig 2006). The present work will further our understanding of how O_2 deprivation affects human adipose tissue lipid storage and mobilization functions and ultimately provide further insight of the metabolic cascade leading to changes in lipid homeostasis in response to a variety of O_2 variations.

1.5 Limitations and delimitations

For *in vitro* studies, the two-dimensional cell culture does not encompass the three dimensional complexity of multi-cellular organisms. With regards to *in vivo* studies, the application of these findings will be limited to healthy males, aged 18-39. Furthermore, the duration of the hypoxia exposure will be restrained to 6 hours to limit the burden, and potential side-effects on the hypoxia naïve participants. Finally, *in vivo* studies will not use stably-labelled tracer infusion to better estimate lipid production and clearance rates.

CHAPTER 2: REVIEW OF THE LITERATURE

2.1 Adipose tissue lipid storage and mobilization functions

2.1.1 General information on triglycerides and adipose tissue

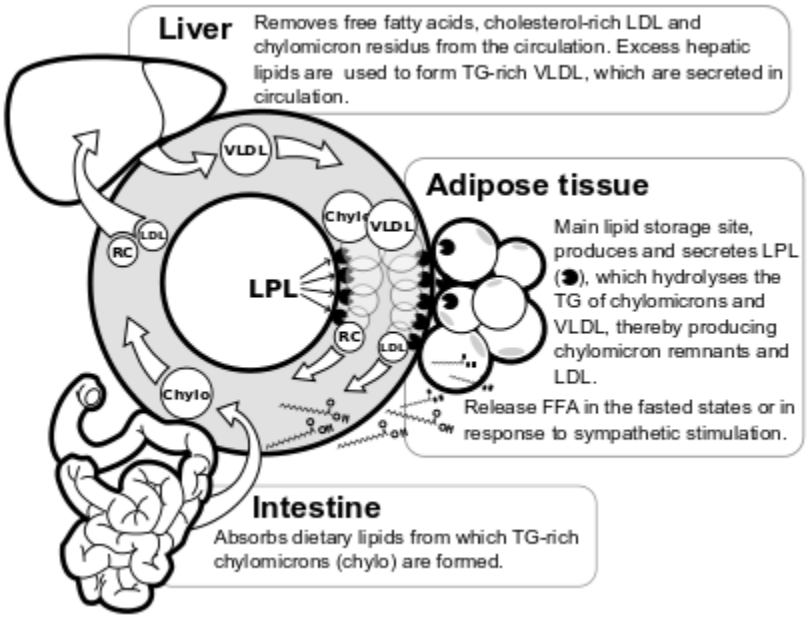

Figure 1. Overview of triglyceride-rich lipoproteins metabolism in postprandial state. LPL: lipoprotein lipase. Chylo: chylomicrons. CM: chylomicron remnants. VLDL: very-low density lipoproteins. TG: triglyceride. LDL: low-density lipoproteins.

Triglycerides

Most energy reserves in the human body are stored as TG, mostly derived from food and composed of glycerol and three fatty acids. There are three main organs that store TG in a regulated way and hydrolyze it to release fatty acids, either for export or for internal consumption. These are, in order of amount of TG typically stored: adipose tissue, skeletal

muscle, and the liver (Frayn 2002). TG are not soluble in plasma, therefore they are transported via the circulatory system in the form of large multi-molecular aggregates, the lipoprotein particles (Fielding and Frayn 1998). The TG-rich lipoprotein particles are called chylomicrons and very-low density lipoproteins (VLDL). Through these, TG are carried from the small intestine and liver to the rest of the tissues. There is a single molecule of apolipoprotein B (ApoB), the main structural surface protein, on each of those lipoproteins, with ApoB-100 for VLDL and ApoB-48 for chylomicrons (Schumaker et al. 1994). ApoB is predictive of atherosclerosis as its overproduction leads to atherosclerosis (Alipour et al. 2008).

Adipose tissue

Adipose tissue is the body's largest energy organ with more than 95% of the body's lipids, stored as TG (**Figure 1)** (Coppack et al. 1994). Less than 0.1% of the body's lipids are in the plasma and there are small amounts of lipids stored in other tissues (liver and muscle) (Coppack et al. 1994, Large et al. 2004). When in dietary excess, TG are mostly stored in subcutaneous adipose tissue since it represents about 85% of all body adipose tissue (Frayn and Karpe 2014).

The adipocytes, the signature cells of adipose tissue, plays a central role in the regulation of TG storage and mobilization (**Figure 2)** (Luo and Liu 2016) because they are able to mobilize NEFA and provide them as systemic energy substrate as compared to non-adipose cells (Frühbeck et al. 2001). A tight control between TG hydrolysis and NEFA esterification for the maintenance of appropriate cellular NEFA concentration must be exerted. This became evident when excessive lipid deposition in non-adipose tissues caused by an impaired capacity of fat cells to buffer NEFA and/or an increased TG mobilization capacity led to lipotoxicity and a greater prevalence

of metabolic disorders (DeFronzo 2004, Lelliott and Vidal-Puig 2004, Slawik and Vidal-Puig 2006).

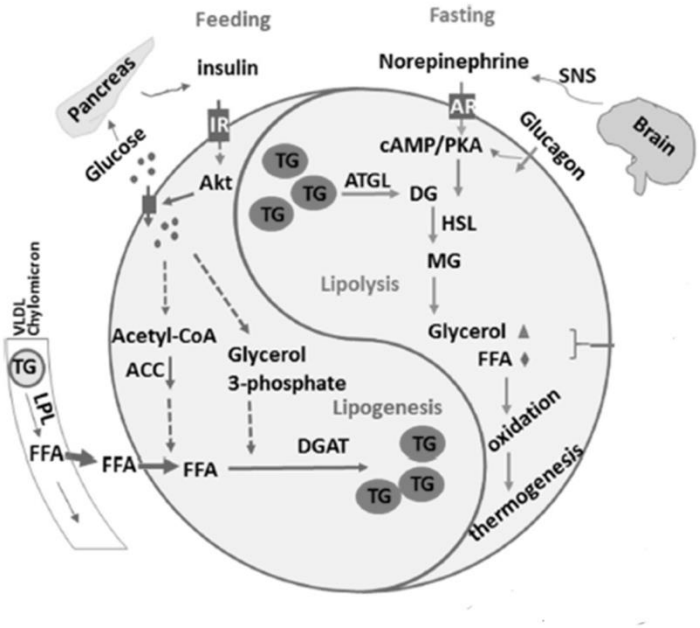

Figure 2. Adipocyte lipid metabolism. TG: triglycerides. HSL: hormone sensitive lipase. ATGL: adipose triglyceride lipase. FFA: free fatty acids. DGAT: diacylglycerol acyltransferase. DG: diglycerides. AR: adrenoceptors. IR: insulin resistance. LPL: lipoprotein lipase. MG: monoglycerides. SNS: sympathetic nervous system. cAMP: cyclic adenosine monophosphate. PKA: protein kinase A. Adapted and modified from Luo et al. (Luo and Liu 2016).

2.1.2 Adipose tissue lipid storage

TG stored in adipose tissue are the body's largest energy reservoir in humans. Adipose tissue stores energy in excess of needs in the form of TG, a process termed lipogenesis that is partly driven by the LPL (Luo and Liu 2016). LPL degrades lipoprotein-bound TG to fatty acids for their subsequent uptake by the adipose tissue where they can be re-esterified into TG (Kersten

2001, Shi and Cheng 2009) through the action of monoacylglycerol acyltransferase (MGAT) and diglyceride acyltransferase (DGAT) activity (Smith et al. 2000, Harris et al. 2011). Compelling evidence indicate that angiopoietin like-4 protein (ANGPTL-4) protein, secreted by adipocytes, inhibits LPL by promoting the conversion of active LPL dimers to inactive LPL monomers (Lichtenstein and Kersten 2010, Kersten 2014). *In vitro* studies have suggested that ANGPTL-4 enzymatically catalyzes the dimer to monomer conversion whereas *in vivo* studies suggest that ANGPTL-4 disables LPL by binding LPL monomers, thereby driving the LPL dimer–monomer equilibrium toward inactive monomers (Lichtenstein and Kersten 2010). Because LPL is a critical determinant of plasma TG clearance and resultant tissue uptake of fatty acids, the activity of LPL needs to be carefully regulated (Kersten 2014).

In addition, adipose tissue can synthesize new fatty acids from other macronutrients, a process called *de novo* lipogenesis (DNL). The regulation of DNL occurs partly at the transcriptional level with the nuclear factor carbohydrate response element-binding protein (ChREBP) responding to glucose availability (Herman et al. 2012) to stimulate the expression of DNL rate-limiting enzymes fatty acid synthase (FAS) and acetyl-coA carboxylase (ACC) ("Adipose tissue de novo lipogenesis" n.d., Shrago et al. 1969, Letexier et al. 2003).

2.1.3 Adipose tissue lipid mobilization

In time of increased metabolic needs, stored lipids can be mobilized by converting adipose tissue TG into fatty acids using a process called lipolysis, which depends mainly on the activation of 2 specific hydrolases, the adipose triglyceride lipase (ATGL) and the hormone-sensitive lipase (HSL) ("Lipolysis, Fat Mobilization, Fatty Acid (beta, alpha, omega) Oxidation, Ketogenesis"

n.d., Lass et al. 2011). Fatty acids derived from lipolysis are released into circulation and delivered to peripheral tissues for sustaining energy demand.

Stimulation of Lipolysis

Catecholamines are one of the hormones that markedly stimulate lipolysis (Dodt et al. 2003, Large et al. 2004, Luo and Liu 2016). First, these hormones are released by the sympathetic nervous system (SNS) which is stimulated during fasting and exercise (Zouhal et al. 2008), and they bind to the β – adrenoceptors (AR) which then activate cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA) (Carmen and Víctor 2006). There are three different β-AR subtypes (β1-ARs, β2-ARs, β3-AR) which activate lipolysis cascade (Enocksson et al. 1995). Catecholamine induced lipolysis is predominantly mediated by β2-ARs which is similar to isoproterenol in healthy subjects (Hansen et al. 1990). While, β1-ARs and β3-AR have minor importance for the stimulation of lipolysis in healthy subjects (Lafontan and Berlan 1993, Enocksson et al. 1995). Second, activated PKA phosphorylates the lipid droplet-associated proteins such as perilipin and cytoplasmic hormone sensitive lipase (HSL) (Marcinkiewicz et al. 2006, Lafontan and Langin 2009). Finally, phosphorylation of perilipin promotes the release of ATGL (Nielsen et al. 2014). In brief, ATGL is responsible for the conversion of TG to diglycerides (DGs) which are hydrolysed by HSL (Zimmermann et al. 2004, Luo and Liu 2016). HSL hydrolyses DGs to monoglycerides (MGs), which mediates the release of free fatty acids and glycerol completing the lipolytic pathways (Haemmerle et al. 2002).

In both men and women, the highest lipolytic activity of catecholamines is found in the visceral fat depot, followed by the abdominal subcutaneous region (the major body fat depot) and the lowest activity in peripheral subcutaneous fat depots (gluteal and femoral) (Leibel et al. 1989).

Other hormones may also stimulate lipolysis in a similar way to catecholamines. These hormones are glucagon, a thyroid stimulating hormone, and cholecystokinin, however, their effects are minimal and the physiological and pathophysiological role in lipolysis is unclear (Marcus et al. 1988, Carlson et al. 1993, Large et al. 2004).

Inhibition of lipolysis

Insulin is the most potent antilipolytic hormone in adipose tissue that stimulates free fatty acid uptake via LPL on circulating TG and increases lipogenesis (Coppack et al. 1989, Large et al. 2004). First, insulin signaling in the adipose tissue involves the activation of the insulin receptor tyrosine kinase, the phosphorylation of insulin receptor substrates, leading to an activation of a phosphatidyl inositol kinase-3 (PIK-3), and the subsequent production of specific phosphoinositides at the plasma membrane (Okada et al. 1994). Second, these phosphoinositides recruits protein kinase B (PKB), where PKB becomes phosphorylated and activates phosphodiesterase-3 (PDE-3) (Lönnroth and Smith 1986, Cheatham and Kahn 1995). Finally, PDE-3 lowers the intracellular level of cAMP and PKA activity and thus completely abolishes the lipolytic effect of human adipose tissue (Hagström-Toft et al. 1995).

Furthermore, α2- AR is the highly potent antilipolytic receptor, which is mainly released during fasting (Lafontan and Berlan 1995, Large et al. 2004). α2- AR is involved in the modulation of lipolysis at rest or when plasma ephinephrine levels are increased (ex. mental stress). However, β2-ARs as catecholamines which stimulate lipolysis, dominates over α2-AR in release during mental stress. In both men and women, the highest antilipolytic activity of insulin is found in the subcutaneous adipose tissue, followed by omental tissue (Bolinder et al. 1983).

Basal lipolysis

In the absence of any stimulatory agents on human fat cells, an *in vitro* spontaneous lipolytic activity is considered as basal lipolysis (Arner 1988, Large et al. 2004). In animal adipose tissue, basal lipolysis is usually undetectable. The rate of basal lipolysis may depend upon the fat cell size: Positive correlation has been found between the basal rate of lipolysis and the fat cell size (Andersson and Arner 1995, Large et al. 2004).

2.1.4 Fasting and postprandial lipid metabolism

As an energy storage organ, adipose tissue stores TG (lipogenesis), synthesizes fatty acid molecules (DNL), and mobilizes fatty acids (lipolysis) (**Figure 2**). Systematically, feeding stimulates the lipogenic pathways, while fasting induces the activation of lipolytic pathway (Luo and Liu 2016).

Fasting lipid metabolism

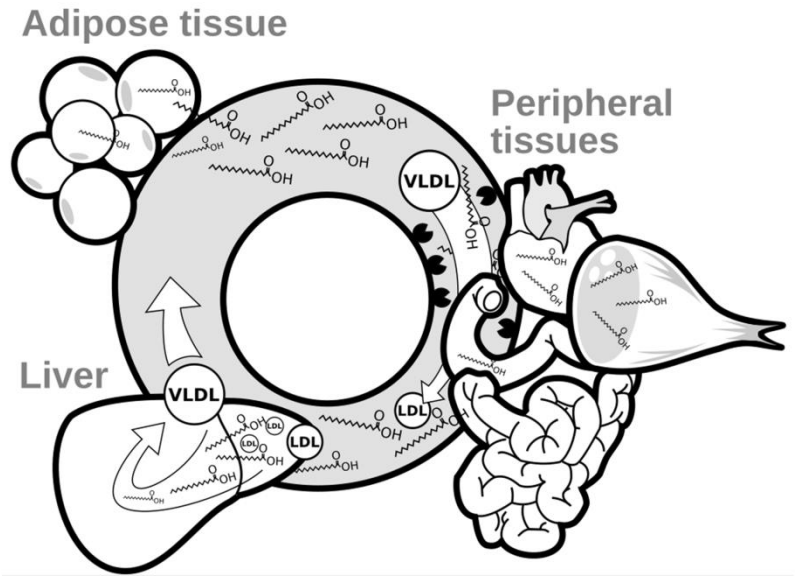

Figure 3. Overview of lipid metabolism in fasting state. VLDL: very-low density lipoproteins. LDL: low-density lipoproteins.

In the transition from fed to 12h fasting, the liver is the master gatekeeper of ingested, mobilized, and *de novo* synthesized lipids, with a far greater capacity than the intestine for storage and maintenance of lipid homeostasis (**Figure 3**) (Xiao et al. 2011). First, NEFA are released from white adipose tissue (WAT) (i.e. lipolysis), which is a critical step aimed at maintaining whole body energy homeostasis in the absence of an external energy supply (Desvergne et al. 2006). NEFA availability, in turn, depends mainly on WAT lipolysis, which is under both sympathetic and hormonal control, with epinephrine and insulin acting respectively as the main systemic activator and inhibitor (Desvergne et al. 2006, Langin 2006). Second, the free fatty acids that are released from WAT are reesterified into TG in the liver and are mobilized to the blood in the form of VLDL (Desvergne et al. 2006). In the fasting state, 70-80% of total liver VLDL-TG

production derives from non-esterified fatty acids (NEFA) (Barrows and Parks 2006). Finally, the peripheral clearance of VLDL-TG, is catalyzed mainly by the LPL and hepatic triglyceride lipase (HL). The lipolytic activity of both can be assessed in post-heparin plasma (Després et al. 1999).

Postprandial lipid metabolism

Following meal ingestion, dietary fat is absorbed by the intestine and TG are released (**Figure 1**). First, TG are transported by lipoproteins such as chylomicrons and VLDL through the intestine and liver in blood circulatory system. Second, insulin is secreted by the β-cells of the pancreas and is influenced by numerous factors such as increased blood glucose (Xiao et al. 2011, Szkudelski and Szkudelska 2015). Finally, insulin activates LPL, which hydrolyses circulating TG, and in turn, releases fatty acids into adipose tissue and their re-esterification into stored TG (Williams 2004).

Insulin secretion results in suppression of lipolysis to basal levels while lipogenesis is stimulated (Williams 2004). Within 24h following a meal while resting, the adipose tissue will store approximately 70% of the chylomicrons fatty acids and the remaining 30% will be oxidized (Jensen 2003). After an overnight fast, the upregulation of LPL in adipose tissue is slower than the appearance of the chylomicrons and the well-timed blood flow response, which leads to less efficient lipid storage in adipose tissue (Ruge et al. 2009). However, in response to a meal, capillaries in the adipose tissue vasodilate to increase the amount of blood in the underlying tissue, resulting in an increased efficiency to manage TG rich lipoprotein (Summers et al. 1996). Furthermore, for subsequent meals, the efficiency of adipose tissue fatty acids uptake increases, as does the LPL action by twofold (Ruge et al. 2009). Since LPL is rate limiting for plasma TG

clearance and adipose tissue uptake of NEFA, the activity of LPL is carefully controlled to adjust NEFA uptake to the requirements of the underlying tissue (Dijk and Kersten 2014).

2.2 Overview of hypoxia

2.2.1 Hypoxia: Obesity

Obesity is the result of an imbalance between energy intake and energy expenditure. When the energy intake exceeds the energy expenditure, there is an energy surplus that is stored mainly in WAT (Landini et al. 2016). It has been suggested that excessive adipose hypertrophy, as observed in animals with obesity, can impair O_2 diffusion in the adipose tissue and lead to adipose tissue hypoxia in animals (Hosogai et al. 2007, Trayhurn and Alomar 2015). Part of the basis for this proposition lies on the limited vascularization of adipose tissue as well as the absence of increase in blood flow in the tissue in obesity (West et al. 1987, Karpe et al. 2002, Kampf et al. 2005, Landini et al. 2016). There are substantial differences between the blood flow levels in the various adipose tissue depots in the body, the visceral depots omental, mesenterial, perirenal, and epicardial having the highest flow levels (Bülow 2001). A considerable difference exists in the O_2 level in specific tissues (**Table 1**). Studies in animals showed that there is a 2- 3 fold reduction in the partial pressure of O_2 (PO_2) in WAT of obese mice, down to 15 mmHg compared with 45-50 mmHg of lean mice (Hosogai et al. 2007, Rausch et al. 2008, Trayhurn and Alomar 2015).

In contrast to the clear evidence for the hypoxia in WAT of animals with obesity, the adipose tissue O_2 tension in humans with obesity is more problematic to conclude due to methodological issues. Earlier studies demonstrated reduced PO_2 at WAT in humans with obesity (Kabon et al. 2004, Pasarica et al. 2009, Trayhurn and Alomar 2015). Since the vascular supply is reduced per

unit adipose mass in humans with obesity; the capillary density is also lower than in the lean. However, recent studies found no evidence in decrease of O_2 levels at WAT as there was no decrease in adipose tissue blood flow in postprandial state for humans having obesity (Goossens et al. 2011, Hodson et al. 2013, Trayhurn and Alomar 2015). At present, there is no evidence why such divergent results have been obtained in humans having obesity.

Table 1. Oxygen level in white adipose tissue and other tissues:

Tissue	**Partial pressure of oxygen, mmHg**
Inspired air (at sea level)	160
Alveolar blood from lungs	104
General tissue oxygenation	40-50
Brain	0.4-8
Retina	2-25
Spleen	16
White adipose tissue, lean mice	47.9
White adipose tissue, obese mice	15.2
White adipose tissue, humans (I) (Pasarica et al. 2009)	lean 55.4/obese 44.7
White adipose tissue, humans (II) (Hodson et al. 2013)	lean 46.8/obese 67.4

Adapted and modified from Trayhurn (Trayhurn 2013). Permission for republication is not required (Appendix D).

2.2.2 Intermittent hypoxia: Obstructive sleep apnea

OSA consists in repeated, momentary cessations of breathing caused by recurrent pharyngeal collapses during sleep. These short periods of breathing cessation are interrupted by short arousals during which pharyngeal muscle tone is increased and breathing is normally resumed (Polotsky et al. 2003). The breathing interruption causes the individuals with OSA to be repeatedly exposed to short periods of hypoxia (also considered intermittent hypoxia), during which blood O_2 saturation decreases (Polotsky et al. 2003). As a result, in peripheral tissues, the required O_2 is not diffused down a pressure gradient into the cells and their mitochondria, where it is used to produce energy in conjunction with the breakdown of glucose, fats and some amino acids (Kumar 2016). According to the International Classification of Sleep Disorder, sleep apnea severity can be categorized based on the respiratory disturbance index (RDI) (Thorpy 2012), which measures the number of respiratory events during sleep, including the number of respiratory-effort related arousals, which are not strictly hypoxia events per se, but rather quick transitions from deep stage of sleep to shallower stage that disrupt sleep. An RDI greater than 15 has been established as the clinical threshold for OSA diagnostic, but in most severe cases of OSA, hypoxia events can occur as often as 40 times per hour.

According to the 2009 Canadian Community Health Survey published by the Public Health Agency of Canada, more than 850,000 Canadian adults were diagnosed with sleep apnea (Government of Canada 2010). While around 3% of Canadian adults were diagnosed with OSA in 2009, it was estimated at that time that a much larger fraction (> 25%) of the Canadian adult population was at risk of suffering from or developing OSA (Government of Canada 2010). Currently, the recognized common risk factors for OSA include excess adiposity (body mass index (BMI) > 35 kg/m^2), age over 50 years and being male. Obesity has been emphasized as one

of the strongest predictors of OSA. It is estimated that 50-60% of all obese individuals have OSA (Resta et al. 2001, Drager et al. 2010) and some experts argues that 90% of the recent increase in OSA diagnosis could be due to the increasing prevalence of obesity (read 2013). A possible explanation for this association could be the fact that adiposity may favor upper airways collapsing during sleep; an observation corroborated by data showing that the frequency of respiratory events during sleep appears to rise with body weight (Ferretti et al. 2001, Newman et al. 2005). The most evident symptom of OSA is excessive daytime sleepiness, but its most important health consequence is a ~2-fold increased risk of developing CVD such as coronary artery disease, heart failure and stroke (Newman et al. 2001, Government of Canada 2010).

2.2.3 Hypoxia: Chronic obstructive pulmonary disease

COPD is a leading cause of global morbidity and is predicted to become the third greatest cause of death worldwide by 2020 (Murray and Lopez 1997). It is the group of lung diseases that includes chronic bronchitis, emphysema, in some cases chronic asthma, and others, which are characterized by restricted airflow. When the supply of O_2 to the lungs is restricted or limited, it increases the risk of individuals with COPD to have hypoxia. Hypoxia is the common condition in individuals with COPD, as such chronic ailments affect the lungs and restrict the supply of O_2 to the tissues and cells in the body. A mounting body of evidence suggests that hypoxemia is more than a signifier of advanced disease (Kent et al. 2011). Chronic hypoxemia as a consequence of COPD may also affect adipose tissue function (van den Borst et al. 2013), such as increased in mRNA expression of inflammatory markers, cluster of differentiation 40 (CD40), mitogen-activated protein kinase 4 (MKK4), and nuclear factor-$_K$B (Tkacova et al. 2013). In

sum, the causes of hypoxia in COPD are that the individuals are unable to breathe properly due to weak lungs and there is a limitation of O_2 supply too (ePainAssist 2017).

About half of all people with severe COPD experience sleep disorders such as OSA or insomnia ("Chronic Obstructive Pulmonary Disease Complications - Chronic Obstructive Pulmonary Disease Health Information - NY Times Health" n.d.). Sleep problems and sleepiness are common in individuals with COPD, partly due to medications used to treat COPD but also due to symptoms. Even COPD patients without OSA may experience a drop in O_2 during sleep ("COPD and Difficulty Breathing" n.d.). In addition, obesity is highly prevalent in individuals with COPD. The prevalence of obesity is the highest among individuals with milder forms of the COPD (Stages 1 and 2), and the lowest in patients with the most severe lung function impairment in Stage 4 (Marquis et al. 2005). It suggests that high adiposity and fat tissue accumulation may impair pulmonary functions (Young et al. 2016).

2.2.4 Hypoxia: High altitude

O_2 mixed with water vapor, diffuses from the breathed air, to arterial blood, where its partial pressure is around 100 mmHg (Brahimi-Horn and Pouysségur 2007, Kumar 2016). In the blood, O_2 is bound to hemoglobin and passively diffuses into the lung alveoli according to a pressure gradient. After reaching peripheral tissues, O_2 diffuses down a pressure gradient into cells and their mitochondria, where it is used to produce energy in conjunction with the breakdown of glucose, fats and some amino acids (Kumar 2016). Hypoxia can result from a failure at any stage in the delivery of O_2 to cells (Brahimi-Horn and Pouysségur 2007).

Altitude is defined by the vertical distance to sea level. Due to the reduction in atmospheric pressure with altitude, O_2 availability is diminished and it represents a stress for the human

organism when not acclimatized. At high altitude (≥3,000 m) (Bärtsch et al. 2008), hypoxia can occur (Surks et al. 1966, Brahimi-Horn and Pouysségur 2007, Bärtsch et al. 2008), which can lead to a decrease in the O_2 content of the human body, a phenomenon called hypoxemia (blood O_2 saturation ≤ 90%). O_2 saturation in healthy individuals varies between 72% and 82% when exposed to 3800m above sea level and everybody will be under hypoxic stress at high altitude (Johnson et al. 2010). High altitude exposure usually occurs mainly under three conditions: exposure to sports and work, mountain trekking, and living at high altitude. Among the modifications in phenotypes due to permanent exposure to high altitude for individual living at high altitudes, it is often reported that cardiovascular adaptation occurs, such as an increase in hemoglobin (West 1990). Given the popularity of mountain trekking and/or high altitude exposure for sports performance to elevations above 4,000 m, and the degree of hypoxemia known to occur at such altitudes, several studies have characterized the physiological consequences of altitude exposure on an important segment of the fuel commonly used during prolonged work that are TG (Whitten and Janoski 1969, Férézou et al. 1988, Young et al. 1989, Leaf and Kleinman 1996, Siqués et al. 2007). It has been reported that individuals living at high altitudes tend to have worse blood lipid profiles and a higher-than-normal prevalence for hypertriglyceridemia, which can increase risk of developing CVD and cause higher mortality (Temte 1996, Mohanna et al. 2006, Hirschler et al. 2012, Gonzales and Tapia 2013). However, due to confounding factors, such as physical activity, and diet; there is less death due to CVD in individuals living at high altitude compared to sea level (Baibas 2005).

2.3 The impact of hypoxia on lipid metabolism and leading to metabolic disorders

Hypoxia may disrupt lipid storage and mobilization and lead to metabolic disorders such as Type 2 diabetes and CVD by impairing adipocytes lipogenesis, by increasing lipolysis, by decreasing lipoprotein clearance, and by rising TG levels.

Impairing human adipocytes lipogenesis and increasing lipolysis

A tight control between human adipocytes lipogenesis and lipolysis must be exerted. Impaired lipogenesis and/or increase lipolysis can overload other organs such as the heart, liver, and skeletal muscles with lipids, which refers to ectopic fat storage. This 'lipotoxic' phenomenon is well recognized to precede the development of metabolic disorders such as diabetes and CVD (DeFronzo 2004, Lelliott and Vidal-Puig 2004, Slawik and Vidal-Puig 2006). Recently, O'Rourke et al. (O'Rourke et al. 2013) observed that severe hypoxia (1% O_2) for 72h inhibits the expression of the lipogenic gene (FAS) without affecting the expression of the lipolytic gene (ATGL) while severe hypoxia (1% O_2) for 24h stimulates basal, but not isoproterenol-induced lipolysis. Famulla et al. (Famulla et al. 2012) showed that chronic exposure to hypoxia (5%, and 10% O_2) increases isoproterenol-stimulated lipolysis and the expression of the lipolytic gene (HSL) but not ATGL on human preadipocytes. These previous studies provide evidence that hypoxia alter the metabolism of human adipocytes *in vitro*. However, these studies differed in terms of *in vitro* modalities of exposure to hypoxia. Consequently, these previous *in vitro* observations regarding the effects of hypoxia on human preadipocytes lipogenesis and lipolysis needs to be further consolidated and validated that could facilitate the treatment and prevention of metabolic disorders such as Type 2 diabetes and CVD.

Decreasing lipoprotein clearance

Recent animal studies demonstrated that chronic intermittent (Drager et al. 2012, Yao et al. 2013) and acute hypoxia (Jun et al. 2012) increased hepatic TG secretion in the fasted state and delay TG clearance in the postprandial state. These changes appear to be caused, in part, by *i*) an increase in lipid influx to the liver due to an increase in adipose tissue lipolysis and by *ii*) a suppression of LPL activity by more than 50%. While the increase in adipose tissue lipolysis has been linked to the increase in sympathetic drive observed during hypoxia; the reduction on adipocyte LPL activity may be explained by the upregulation of an important post-translational repressor of LPL, angiopoietin like-4 protein (ANGPTL-4), during hypoxia exposure (Drager et al. 2012). ANGPTL-4 seems to increase by 2 to 4.5 fold in WAT, but not in cardiac skeletal muscle or liver in response to decrease in LPL activity (Drager et al. 2013).

At the cellular level, the HIF-1 acts as the master O_2 sensor and mediates cellular responses to hypoxia. HIF-1 is involved in the expression of more than 60 genes involved in glucose metabolism, angiogenesis, and cell death, among others (Semenza 1999, 2014, 2017). Previous *in vitro* work has demonstrated that the expression of ANGPTL-4 gene is under the control of HIF-1 (Zhang et al. 2012) and is significantly induced in a dose-dependent manner on differentiated human preadipocytes exposed to low O_2 tension (Wood et al. 2011). In humans, the gene encoding ANGPTL-4 is predominantly detected in adipose tissue and liver (Kersten et al. 2009). Evidence strongly suggests that ANGPTL-4 plays a major role in the regulation of lipid metabolism (Lichtenstein and Kersten 2010) by acting as an inhibitor of the enzyme LPL, thereby suppressing the clearance of TG-rich lipoproteins and raising plasma TG levels (Sukonina et al. 2006). However, this hypothesis remains to be tested in humans.

In turn, these changes in adipose tissue lipolysis and LPL activity by hypoxia could lead to adverse alteration of the blood lipid and lipoprotein profile, by increasing TG levels and inhibiting VLDL clearance and causing hypertriglyceridemia and dyslipidemia, which could contribute to the increased CVD risk. One of the circumstances that affect the supply of O_2 is OSA where individuals with OSA experience short periods of hypopnea, inducing intermittent hypoxia-hypercapnia/normoxia cycles. It has been shown that individuals with OSA showed increased TG (by ~30%) compared to individuals without OSA (Newman et al. 2001). Although continuous positive airway pressure (CPAP) treatment had been reported as being efficient at partially normalizing blood lipid and lipoprotein profile in individuals with OSA, it remains an expensive treatment that a significant portion of the population cannot afford or considers too uncomfortable to use during sleep (Sawyer et al. 2011). A better appreciation of how intermittent hypoxia, a simulation model of OSA, affects human adipose tissue lipolysis and LPL activity could facilitate the treatment and prevention of metabolic disorders such as Type 2 diabetes and CVD. However, the effects of intermittent hypoxia, a simulation model of OSA, on lipid and adipose tissue metabolism have never been investigated in humans (**Figure 4**).

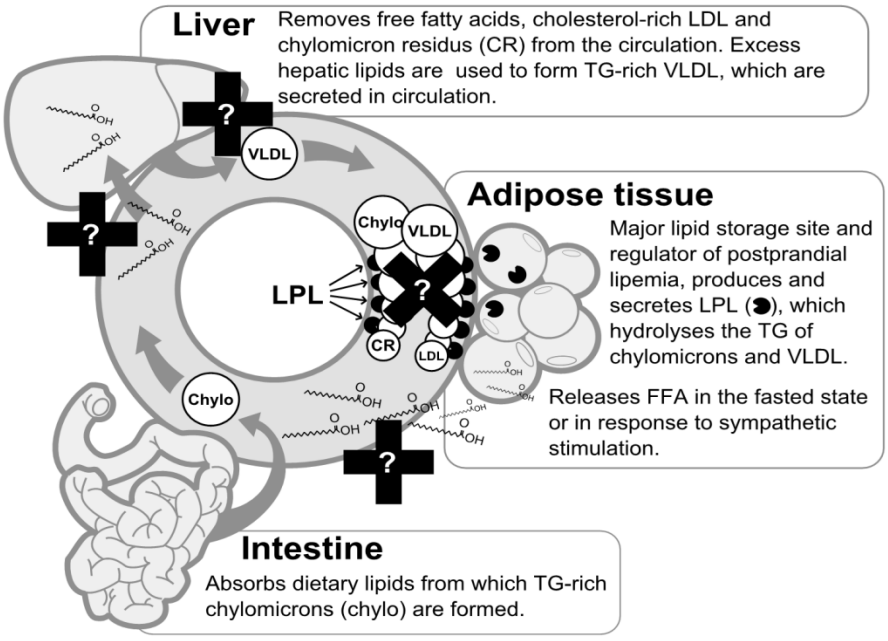

Figure 4. Summary of possible adverse effects of intermittent hypoxia on postprandial lipemia. LPL: lipoprotein lipase. Chylo: chylomicrons. CR: chylomicrons remnants. VLDL: very-low density lipoproteins. TG: triglycerides. LDL: low density lipoproteins.

Rising TG levels

Proper TG metabolism is critical for global energy homeostasis. It is thought that impaired lipid storage and over exposition of organs to circulating lipids can lead to ectopic fat storage and lipotoxicity, which have been linked to impaired insulin secretion and reduced peripheral insulin signaling as well as the development of chronic diseases such as Type 2 diabetes and cardiovascular disease (CVD) (Kalofoutis et al. 2007, Miller et al. 2011). Fasting circulating TG concentrations reflect the balance between hepatic VLDL-TG secretion and peripheral VLDL-TG clearance (Parks et al. 1999, Barrows and Parks 2006). In the fasting state, 70-80% of total

liver VLDL-TG production derives from NEFA and NEFA availability, in turn, depends mainly on WAT lipolysis (Barrows and Parks 2006). The peripheral clearance of VLDL-TG, on the other hand, is catalyzed mainly by the LPL and the HL. Animal studies have already shown that acute (Jun et al. 2012, 2013) and prolonged hypoxia (Drager et al. 2012, Yao et al. 2013) increases adipose tissue lipolysis and decreases LPL activity, which suggests that hypoxia may increase NEFA delivery to the liver and increase plasma TG concentrations in the fasting state. However, it is still not clear whether these observations also occur *in vivo* in humans in the fasting state.

Over the years, the effect of prolonged hypoxia exposure is not consistent on fasting TG levels in humans. An earlier terrain study by Whitten et al. (Whitten and Janoski 1969) showed a +44% increase in TG levels after 9 days of exposure at 4265m. Another terrain study performed by Siques et al. (Siqués et al. 2007) reported a +47% increase in TG levels following 8 months of exposure to 3550m. Conversely, Férézou et al. (Férézou et al. 1988) showed that fasting TG levels measured in 8 individuals decreased by -42% after 33 days of exposure to 4800m. These discrepant results on the effects of terrestrial high altitude exposure on TG levels may be explained by confounders such as exercise, weight loss, cold exposure, and/or perturbed nutritional intake related to ascent. To control for these potential confounders on blood lipid levels, Férézou et al. (Férézou et al. 1993) transferred 6 individuals living at sea level to 4350m by helicopter and showed that after a 7-day sojourn, fasting TG levels dropped by -26% while body weight and nutritional intake remained stable. Using a hypobaric chamber, 6 men participating in the Operation Everest II study showed an +81% increase in fasting TG levels while exposed to 40 days of simulated altitude through a progressive decreased partial pressure

of air equivalent, at the end, to 282 Torr and during which they have lost ~9% of their initial body weight (Young et al. 1989).

Similarly, the effect of acute exposure to hypoxia is not consistent on TG levels in humans and animals. Less severe hypoxia conditions (FiO_2 = 16%, equivalent to 2200m altitude) for a significantly shorter duration (2 hours) reported no change in fasting plasma TG levels in humans (Leaf and Kleinman 1996). These observations seem conflicting with emerging evidence from animal studies showing a strong and rapid deleterious impact of hypoxia on lipid metabolism (Jun et al. 2012, 2013). Discrepancies in TG response to hypoxia may be related to the thermal condition during which hypoxia occurs. Jun et al. (Jun et al. 2013) have shown that, in mice, elevation in TG levels in response to hypoxia occurs in cold conditions (22 °C) but not at thermoneutrality (30 °C). They showed that cold up-regulates TG uptake in several tissues, namely brown adipose tissue, favoring sustained low TG levels in cold exposed rodents. At thermoneutrality, they demonstrate that mice TG levels are considerably higher than those of counterparts kept at 22 °C and that hypoxia no further increased plasma TG in these conditions. Whether a similar cold-hypoxia interaction is species-specific or occurs also in humans is unknown and warrant further research. However, recent experiments done on cold-acclimated humans showed no effect of a 5-hour cold exposure both on postprandial TG levels and dietary TG clearance rate (Blondin et al. 2017), suggesting that the lipid response to cold exposure is not as strong in humans as in rodents.

In conclusion, despite studies having reported conflicting results regarding the effect of hypoxia on plasma TG concentrations in humans, which could be due to the poor level of control for confounding factors such as physical activity, and diet (Whitten and Janoski 1969, Férézou et al. 1988, Young et al. 1989, Leaf and Kleinman 1996, Siqués et al. 2007), and relatively strong

evidence from animal study supporting an important deleterious impact of acute hypoxia on TG depending upon environmental conditions, namely temperature (Jun et al. 2012, 2013); it is not clear whether a deleterious impact on blood lipid profile exists when fasting healthy men are exposed to hypoxia.

2.4 Effects of hypoxia in other parts of human body

2.4.1 Nervous system

Hypoxia activates the SNS by circulating catecholamine levels using microneurography (Hansen and Sander 2003). The technique, called microneurography, is used to measure SNS activity and consists of measuring the nervous system by burst per minute directly in a tissue, such as muscle. Studies using microneurography on people exposed to hypoxia report a threefold increase in sympathetic activity (Hansen and Sander 2003). Therefore, it can be concluded that hypoxia exposure is associated with a shift in sympathovagal balance toward heightened SNS activity (Hansen and Sander 2003, Louis and Punjabi 2009, Jun et al. 2012). Activation of the SNS leads to an increase in catecholamine efflux by the adrenal medulla (Mesarwi et al. 2015). Catecholamine stimulates glucagon secretion, activates glycogenolysis and gluconeogenesis in the liver, and causes the breakdown of muscle glycogen and adipose tissue TG. Catecholamines also inhibit insulin secretion and insulin-mediated glucose uptake by the skeletal muscle (Mesarwi et al. 2015). It was first believed that adipose tissue lipolysis is mediated only by circulating catecholamines. However, it is now reported that adipose tissue is directly innervated by SNS and thus does not require the circulating catecholamines to start lipolysis (Youngstrom and Bartness 1995). During hypoxia, circulating catecholamines, through their action on α-ARs,

promote glycogenolysis and inhibit pancreatic insulin secretion, to cause hyperglycemia and glucose intolerance (Jun et al. 2014).

2.4.2 Cardiovascular system

During low O_2 exposure, heart rate is inversely proportional to O_2 availability (Mazzeo et al. 1994). Reduction in the systemic partial pressure of O_2 leads to an increase in heart rate. The principal acute effect of hypoxia is a blood redistribution among the limbs which causes the heart rate to increase and stroke volume to decrease, without increasing blood pressure in order to maintain mean arterial pressure (Sagawa et al. 1993). However, under exposure to continuous low oxygenation (fraction of inspired O_2 (FiO_2)$\approx$ 0.12) for 2 to 6 days, blood pressure is increased (Cornolo et al. 2004, Peltonen et al. 2012). The increase in heart rate is generally 10-15 beats per minute (BPM) faster when exposed to hypoxia (12.5% of O_2) as compared to normoxia (20.93% O_2) (Hooper and Mellor 2011).

2.4.3 Substrate oxidation rate

The human body mostly relies on carbohydrate (CHO) and lipid substrate for sustaining its energy production (ATP) (Young 1990). To sustain energy demand, lipids come mainly from adipose tissue lipolysis while CHO come from glycogenolysis of the liver. Lipids, through oxidative phosphorylation, have the power to generate a lot of ATP compared to CHO (Young 1990). Brooks et al. (Brooks et al. 1991) and Roberts et al. (Roberts et al. 1996) reported increases in CHO oxidation rate and decreases in lipid oxidation rate at rest in individuals

chronically exposed (21 days) to high altitude (4300m) and no apparent effects on fatty acid oxidation when acutely exposed to hypoxia.

CHAPTER 3: METHODS AND RESULTS

3.1 Thesis article #1: Effects of Different Oxygen Tensions on Differentiated Human Preadipocytes Lipid Storage and Mobilization Functions

At the time of thesis submission, the data collection of Study I was just finished, so Study I is in progress of manuscript writing and has been formatted according to the thesis. The partial data of Study I are also present in Study II, which was published and can be found in Appendix B.

Effects of Different Oxygen Tensions on Differentiated Human Preadipocytes Lipid Storage and Mobilization Functions

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Abstract

Some studies suggest that adipose hypertrophy, as observed in individuals with excess adiposity, can impair oxygen (O_2) diffusion in the adipose tissue and cause adipose tissue hypoxia. The present study aimed at characterizing the effects of hypoxia on adipocyte lipid storage and lipid mobilization functions. Human preadipocytes were exposed to different O_2 tensions (severe hypoxia 3% O_2, mild hypoxia 10% O_2, and control 21% O_2) either acutely for 24h after differentiation (acute exposure) or during differentiation (14d, chronic hypoxia). Lipoprotein lipase (LPL) activity, basal and isoproterenol-stimulated lipolysis, and expression of genes involved in lipid storage and lipid mobilization were assessed. Both acute and chronic exposure to hypoxia inhibited LPL dose-dependently ($p<0.05$). Acute exposure to mild hypoxia stimulated the expression of lipid storage genes ($p<0.05$) while chronic exposure to severe hypoxia inhibited the expression of genes involved in lipid storage and lipid mobilization ($p<0.05$). Acute exposure to hypoxia had a concentration-dependent stimulating effect on basal ($p<0.05$), but not isoproterenol-induced lipolysis. Conversely, chronic exposure to hypoxia, both mild and severe, had an inhibiting effect ($p<0.05$) on isoproterenol-induced, but not basal lipolysis. In conclusion, both acute and chronic hypoxia (3% and 10% O_2) affects adipocyte lipid storage and mobilization functions that could favor ectopic fat deposition.

Keywords: hypoxia, adipose tissue, lipid storage, lipid mobilization, metabolic disorders.

Introduction

Restriction in oxygen (O_2) supply and/or increased O_2 consumption, such as in the cases of chronic obstructive pulmonary disease (COPD) (Raguso et al. 2004, Baldi et al. 2010), obstructive sleep apnea (OSA) (Young et al. 2002) and high altitudes (Siqués et al. 2007), can lead to oxyhemoglobin desaturation and tissular hypoxia. It is also debated that excessive adipose hypertrophy, as observed in individuals with obesity, can impair O_2 diffusion in adipose tissue and cause adipose tissue hypoxia in humans (Pasarica et al. 2009, Goossens et al. 2011, Trayhurn and Alomar 2015).

Adipocyte, the signature cells of adipose tissue, plays a central role in the regulation of lipid storage and lipid mobilization (Luo and Liu 2016). Adipocytes store energy in excess of needs in the form of triglycerides (TG), a process termed lipogenesis that is partly driven by the lipoprotein lipase (LPL) (Luo and Liu 2016). LPL degrades lipoprotein-bound TG to fatty acids for their subsequent uptake by the adipocytes where they can be re-esterified into TG through the action of acyltransferases (MGAT (monoacylglycerol acyltransferase activity) and DGAT (diacylglycerol acyltransferase activity)) (Shi and Cheng 2009, Luo and Liu 2016). In addition, adipocytes can synthesize new fatty acids from other macronutrients, a process called *de novo* lipogenesis (DNL). Part of DNL regulation occurs at the transcriptional level through the nuclear factor carbohydrate response element-binding protein (ChREBP), which stimulates the expression of DNL rate-limiting enzymes acetyl-coA carboxylase (ACC) and fatty acid synthase (FAS) in response to increase in glucose availability (Shrago et al. 1969, Herman et al. 2012). In time of increased metabolic need, stored lipids can be mobilized by converting adipocytes TG into fatty acids using a process called lipolysis, which depends mainly on the activation of 2 specific hydrolases, the adipose triglyceride lipase (ATGL) and the hormone-sensitive lipase

(HSL) (Lass et al. 2011). Fatty acids derived from intracellular lipolysis are released into circulation and delivered to peripheral tissues for sustaining energy demand.

A tight control between adipocytes lipid storage and mobilization functions must be exerted. Impaired lipid storage and/or excessive mobilization of lipid stores can lead to lipid storage in other organs such as the heart, liver, and skeletal muscles, a process termed ectopic fat storage. This 'lipotoxic' phenomenon is increasingly recognized to precede the development of metabolic disorders such as diabetes and cardiovascular diseases (CVD) (DeFronzo 2004, Lelliott and Vidal-Puig 2004). Hypoxia has recently been proposed as a potent perturbator of human adipose tissue metabolism and better understanding the effect of hypoxia on adipose physiology could facilitate the treatment and prevention of metabolic disorders such as Type 2 diabetes and CVD.

To date, a paucity of *in vitro* studies tried to determine the effects of hypoxia on human preadipocytes lipid storage and mobilization functions. Recently, we have reported that acute exposure to severe hypoxia (3% O_2) reduces adipose tissue LPL activity of differentiated human preadipocytes (Mahat et al. 2016). O'Rourke et al. (O'Rourke et al. 2013) observed that severe hypoxia (1% O_2) stimulates basal, but not isoproterenol-induced lipolysis after 24h, and inhibits the expression of the lipogenic gene (FAS) without affecting the expression of the lipolytic gene (ATGL) after 72h. On the contrary, Famulla et al. (Famulla et al. 2012) showed that chronic exposure to hypoxia (5%, and 10% O_2) increases isoproterenol-stimulated lipolysis and the expression of HSL (but not ATGL) in human preadipocytes. These studies provide evidence that hypoxia alter the metabolism of human adipocytes *in vitro*. However, these studies differed in terms of hypoxia exposure modalities. In order to consolidate/validate and expand previous observations regarding the effects of hypoxia on differentiated human preadipocytes *in vitro*, we investigated the effects of different O_2 tensions (severe hypoxia 3% O_2, mild hypoxia 10% O_2,

and control 21% O_2) for different durations, i.e. 24h (acute) and 14 days (chronic) on LPL activity, lipolysis and the expression of several genes involved in lipid storage and lipid mobilization. We hypothesized that hypoxia (3% and 10% O_2) dose-dependently inhibits LPL activity and reduces the expression of genes involved in lipid storage while stimulating lipolysis and increasing the expression of genes involved in the lipolytic pathway in differentiated human preadipocytes.

Methods

Culture of human preadipocytes

Cryopreserved subcutaneous abdominal preadipocytes from two Caucasian females (average age: 39 y; mean body mass index: 22.74 kg/m^2) were commercially obtained from Zen-Bio (NC, USA). Cells were plated and differentiated for 14-days according to the manufacturer's instructions. For chronic hypoxia exposures, culture media were partially changed every 48-72h over the 14-d differentiation period to prevent pH reduction likely due to lactic acid accumulation. Fourteen days post-induction, cells were either directly assayed (chronic exposure) or moved to the proper oxygen tension for 24h (acute exposure) before being assayed.

LPL activity

Cells were washed 3 times with PBS and incubated for 30 minutes in BM-1 containing 100 U/ml heparin at the proper O_2 conditions. LPL activity was measured using the EnzChek Lipase Substrate (Thermo Fisher Scientific), a fluorescent triacylglycerol analog. Fluorescence emission was followed over 1 hour at 37°C. Average blank-adjusted relative fluorescence units (RFU) are reported here. All samples from an identical experiment were assessed simultaneously, alongside positive controls containing bovine LPL (Basu et al. 2011, Mahat et al. 2016).

RNA isolation and RT-PCR

Cells were washed 3 times with PBS and lysed with RLT buffer (QIAGEN) containing 10% β-mercaptoethanol (Mahat et al. 2016). Total RNA was extracted from cell lysates using QIAGEN RNeasy Mini Kits, following the manufacturer's instructions. Complementary DNA was prepared using QIAGEN Reverse Transcriptase Kit. Expression of genes involved in lipid storage (ChREBP, ACC, FAS, diacylglycerol acyltransferase activity 1 (DGAT1), and diacylglycerol acyltransferase activity 2 (DGAT2)) and lipid mobilization (ATGL and HSL) were determined by real-time PCR (RT-PCR) using Eva Green Master Mix (Montreal Biotech, Qc, Canada) on a Rotor-Gene 3000 (Corbett or QIAGEN?) using Quantitect primers (forward and reverse) from QIAGEN, with β-actin serving as the reference gene. Delta-delta CT (cycle threshold) analyses were conducted using the Rotor-Gene 6000 software version 1.7.

Lipolysis

Cells were washed 3 times with PBS and incubated at 37°C for 3 hours in BSA-Krebs-Ringer buffer with or without isoproterenol (10 μM). Lipolytic rate was determined by glycerol quantification using bioluminescence, as described by Mauriège et al. (Mauriège et al. 1999). Results are presented as μmol of glycerol released per well per 3 hours.

Statistical Analysis

Data are expressed as mean ± standard deviation. Means were calculated from three replicates within each experimental group. Differences between acute or chronic exposure to different O_2 tensions were analyzed using one-way analysis of variance. For post hoc analysis, data were analyzed using Tukey's test. A level of significance of $p<0.05$ was considered statistically significant. All analyses were performed using SPSS Statistics 12.0, SPSS Inc., Illinois, USA.

Results

LPL Activity and TG Content in Response to Hypoxia

Both acute and chronic hypoxia induced a concentration-dependent inhibiting effect on differentiated preadipocytes LPL activity (one-way ANOVA, acute p=0.001, chronic p=0.001) (**Figure 1A)**.

Acutely, hypoxia reduced the TG content of mature adipocytes only at 3% (vs 21% p=0.018, vs 10% p=0.039) while chronic hypoxia had a O_2 dose-dependent lowering effect on TG content (one-way ANOVA, p=0.001) (**Figure 1B**).

Effects of Hypoxia on the Expression of Lipogenic Genes

Lipogenic gene expression levels of differentiated preadipocytes in response to acute (24h) or chronic (14d) mild (10% O_2) and severe (3% O_2) hypoxia are illustrated in **Figure 2**. Acute hypoxia, both at 3% and 10% O_2, significantly increased the expression of ChREBP mRNA (p=0.001) as compared to levels observed at 21% O_2. No significant difference in ACC gene expression levels was observed between conditions. FAS (vs 21%, p=0.021) and DGAT2 (vs 21% p=0.006) mRNA expression levels were only significantly increase under acute mild hypoxia. Chronic hypoxia induced a significant gene expression reduction of ChREBP (vs 21% p=0.001, vs 10% p=0.001), ACC (vs 21% p=0.001, vs 10% p=0.011), FAS (vs 21% p=0.030, vs 10% p=0.047) and DGAT2 (vs 21% p=0.001, vs 10% p=0.001) under severe chronic hypoxia (3% O_2) only. Chronic hypoxia had a concentration-dependent repressing effect on DGAT1 mRNA expression (one-way ANOVA, p=0.001)**.**

Effects of Hypoxia on Lipolysis and the Expression of Lipolytic Genes

The effects of different hypoxic modalities on lipolysis of differentiated preadipocytes are summarized in **Figure 3**. Acute hypoxia had a concentration-dependent stimulating effect on

basal lipolysis (one-way ANOVA, p=0.001, **Figure 3A)** while chronic hypoxia had no apparent effect on basal lipolysis. On the contrary, acute hypoxia, both mild and severe, had no significant effect on isoproterenol-induced lipolysis while chronic hypoxia, both at 3% and 10% O_2, significantly inhibited isoproterenol-induced lipolysis (one-way ANOVA, p=0.002, **Figure 3B)**.

Figure 4 illustrates the effects of hypoxia on the expression of selected lipolytic genes. Acute hypoxia had no effects on ATGL and HSL mRNA expression, while significant reductions were observed in ATGL (vs 21% p=0.001, vs 10% p=0.001) and HSL (vs 21% p=0.014, vs 21% p=0.006) mRNA expression upon chronic exposure to severe hypoxia (3% O_2).

Discussion

The goal of the present study was to investigate the lipogenic and lipolytic responses of human differentiated preadipocytes exposed acutely and chronically to both mild and severe hypoxia. The three O_2 concentrations used in the study were chosen based on following arguments. First, cell culture has traditionally been done under 21% O_2, so this O_2 concentration has been used for the control condition (Famulla et al. 2012, O'Rourke et al. 2013). We used 10% O_2 as a mildly hypoxic condition since it has been reported that the real O_2 tension in human adipose tissue is closer to 10% O_2 (Goossens et al. 2011, Trayhurn and Alomar 2015). Alternatively, 10% O_2 could also be considered a more physiologically relevant control condition. Finally, we chose 3% O_2 as the severe hypoxic condition based on the fact that other studies have used O_2 concentrations ranging from 1-5% O_2 to study the effects of hypoxia *in vitro* (Famulla et al. 2012, O'Rourke et al. 2013). We hypothesized that, in differentiated human preadipocytes, hypoxia would dose-dependently inhibits LPL activity and reduces the expression of genes involved in lipid storage while stimulating lipolysis and increasing the expression of genes

involved in the lipolytic pathway. Our observations suggest that both mild and severe hypoxia, acutely and chronically, do appear to significantly affect human adipose tissue lipid metabolism, although in a slightly different manner.

Effects of Hypoxia on Differentiated Human Preadipocytes Lipid Storage Functions

Both the acute and chronic exposure to hypoxia had a concentration-dependent inhibiting effect on LPL activity (**Figure 1)**. These results confirm our previous results regarding exposure of hypoxia on LPL activity (Mahat et al. 2016). The reduction on adipocyte LPL activity may be explained by the upregulation of an important post-translational repressor of LPL, angiopoietin like-4 protein (ANGPTL-4), during hypoxia exposure (Drager et al. 2012, Makoveichuk et al. 2013).

Consistent with the decrease in LPL activity, chronic exposure to severe hypoxia induced a decrease in the expression of several lipogenic genes, namely ChREBP, ACC, FAS, DGAT1 and DGAT2 (**Figure 2**). It therefore appears that sustained severe hypoxia significantly reduces the potential for TG synthesis and thus energy storage. This notion is consistent with the significantly lower cellular TG content observed in these conditions (**Figure 1)**. More intriguing however is the increased expression of some lipogenic genes, namely ChREBP, FAS ad DGAT2, after acute mild hypoxia (10% O_2), which was not observed after acute severe hypoxia (except for ChREBP) nor after chronic hypoxia. The greater expression in lipogenic genes after 24h of mild hypoxia could be the result of the increase in ChREBP expression, which in turn could be due to an increase in glucose uptake under acute hypoxia. Wood et al. (Wood et al. 2007) indeed demonstrated that acute hypoxia increase over 8-fold the transcription of glucose transporter 1 (GLUT1), glucose transporter 3 (GLUT3) and glucose transporter 5 (GLUT5) genes in human adipocytes. A greater density of these glucose transporters at the membrane could increase

cytosolic glucose concentration, induce the nuclear translocation of ChREBP and, in turn, the transcription of lipogenic genes.

On the contrary, our results indicate that any greater hypoxic challenge, in terms of severity or duration, has no effect or even decrease the lipogenic potential of human adipocytes. O'Rourke et al. (O'Rourke et al. 2013) similarly observed that a 3 day exposure to severe hypoxia (1% O_2) inhibits FAS mRNA expression by 20-30% in human visceral adipocytes. While O'Rourke et al. (O'Rourke et al. 2013) attributed part of the reduction in lipogenesis to a decrease in glutamine metabolism and hexosamine production; the physiological mechanisms responsible for the reduced lipogenic potential in response to severe or sustained hypoxia are largely unknown and warrant further studies. However, it could be hypothesized that lipogenesis, being an anabolic process, requires ATP and therefore O_2. It is not unlikely that the shutdown of the ATP-consuming lipogenesis pathway in response to low O_2 condition occurs to preserve energy for cell survival (Liu et al. 2006).

In sum, our observations suggest that hypoxia dose-dependently inhibit LPL activity but that acute mild hypoxia seems to partly stimulate the *de novo* lipogenic pathway while severe or sustained hypoxia appear to repress *DNL*. These observations suggest that chronic hypoxia could impede the proper storage of circulating TG as well as the conversion of non-lipidic energy substrate to fatty acids. In the long-term this could contribute to insulin resistance by impairing the adipose tissue non-oxidative glucose disposal and/or expose non-adipose organs such as the heart, liver and skeletal muscles, to an excess of lipoprotein-bound TG and increase the risk of developing metabolic disorders, such as Type 2 diabetes and CVD.

Effects of Hypoxia on Differentiated Human Preadipocytes Lipolytic Functions

We also compared the effects of acute and chronic hypoxia, both mild and severe, on lipolytic functions of differentiated human subcutaneous preadipocytes. Acute hypoxia had a concentration-dependent stimulating effect on basal but not isoproterenol-stimulated lipolysis (**Figure 3)**. This is consistent with O'Rourke et al. (O'Rourke et al. 2013) who showed an increased basal lipolysis and an absence of change in stimulated lipolysis following a 24h exposure to 1% O_2 of isolated visceral and subcutaneous adipocytes. Interestingly, increased basal but normal isoproterenol-stimulated lipolysis has also been observed in isolated adipocytes from individuals with obesity (Bougnères et al. 1997, Mauriège et al. 1999, Large et al. 2004, O'Rourke et al. 2013). It is therefore possible that hypoxia may play a role in the development of hypertrophied adipocyte lipolytic phenotype. Adipocyte lipolytic response is well recognized to be influenced by cell size, with large adipocytes displaying higher lipolytic rates (Arner and Ostman 1978). Although cell size was not measured in the present study, TG content was decreased dose-dependently by hypoxia, which strongly suggests that hypoxia may have induced a decrease rather than an increase in cell size. Because of this, it is unlikely that the effect of hypoxia on basal lipolysis may be attributable to an increase in adipocyte size. Other factors that may explain the pattern of lipolytic response to acute hypoxia include the inhibition of hexosamine biosynthesis, as proposed by O'rourke et al. (O'Rourke et al. 2013) as well as an increase in the production of the inflammatory markers that is tumor necrosis factor alpha (TNF-α) (Green et al. 2004, Ye et al. 2007, Bézaire et al. 2009). Altogether, these observations suggest that acute hypoxia increase basal lipolysis without affecting the adipocyte response to the β-adrenergic receptor agonist, isoproterenol.

As for lipogenesis, the effects of chronic hypoxia on lipolysis somewhat diverge from the effects of acute hypoxia. Chronic hypoxia reduced the lipolytic response to isoproterenol, without

affecting basal lipolysis (**Figure 3)**. In the case of chronic severe hypoxia, the reduction in isoproterenol-stimulated lipolysis is concordant with the observed significant decrease in ATGL and HSL gene expression (**Figure 4)**. On the other hand, no change in ATGL or HSL expression can explain the significant decrease in catecholamine stimulated lipolysis caused by chronic mild hypoxia. The few studies that studied the effect of chronic hypoxia on isoproterenol-stimulated lipolysis are conflicting. While Famulla et al. (Famulla et al. 2012) showed that chronic exposure to hypoxia *in vitro* (5% and 10% O_2) increases isoproterenol-induced lipolysis in human preadipocytes, de Glisezinski et al. (de Glisezinski et al. 1999) showed that human adipocytes exposed to high altitude (7% O_2, for 31 days) had decreased isoproterenol-stimulated lipolysis rate *ex vivo*. While it is hard to reconcile these observations, it has been suggested that acute induction of lipolysis by β-adrenergic stimulation increases O_2 consumption (Yehuda-Shnaidman et al. 2010). Since lipolytic rates were assessed under hypoxia, it is possible that the lack of O_2 *per se* may have blunted the ability of adipocytes to increase their lipolytic rate. Interestingly, adipocytes from individuals with severe obesity also respond poorly to catecholamine stimulation, which could possibly be explained by a decrease in β2-adrenergic receptor density (Reynisdottir et al. 1994, Large et al. 2004). Further studies will need to be conducted to elucidate how chronic hypoxia can reduce isoproterenol-induced lipolysis without affecting basal lipolysis and to examine if the same mechanisms can explain the catecholamine resistance observed in obese adipocytes.

Conclusions

The present study demonstrates that 1) hypoxia dose-dependently inhibit LPL activity; 2) acute mild hypoxia seems to partly stimulate the *de novo* lipogenic pathway while severe or sustained

hypoxia appear to repress DNL; 3) acute hypoxia have a concentration-dependent stimulating effect on basal but not isoproterenol-stimulated lipolysis; and 4) chronic hypoxia inhibits isoproterenol-stimulated but not basal lipolysis. Therefore, both acute and chronic hypoxia appears to affect human adipose tissue lipid storage and mobilization functions, although in a different manner. Our observations suggest that hypoxia may impair adipose tissue lipid metabolism and expose other organs such as the heart, liver, and skeletal muscles to an excess of lipids and favor the risk of developing metabolic disorders, such as Type 2 diabetes and CVD.

Author Contributions

All authors had full access to all of the data in the study and gave final approval to the submitted version. Study design and conduct: PI, JFM, and BM. Data collection and analysis: JFM, PI, and BM. Data interpretation: PI, JFM, BM. Manuscript writing: BM, JFM and PI.

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Competing interests

The authors declare that they have no competing financial interest.

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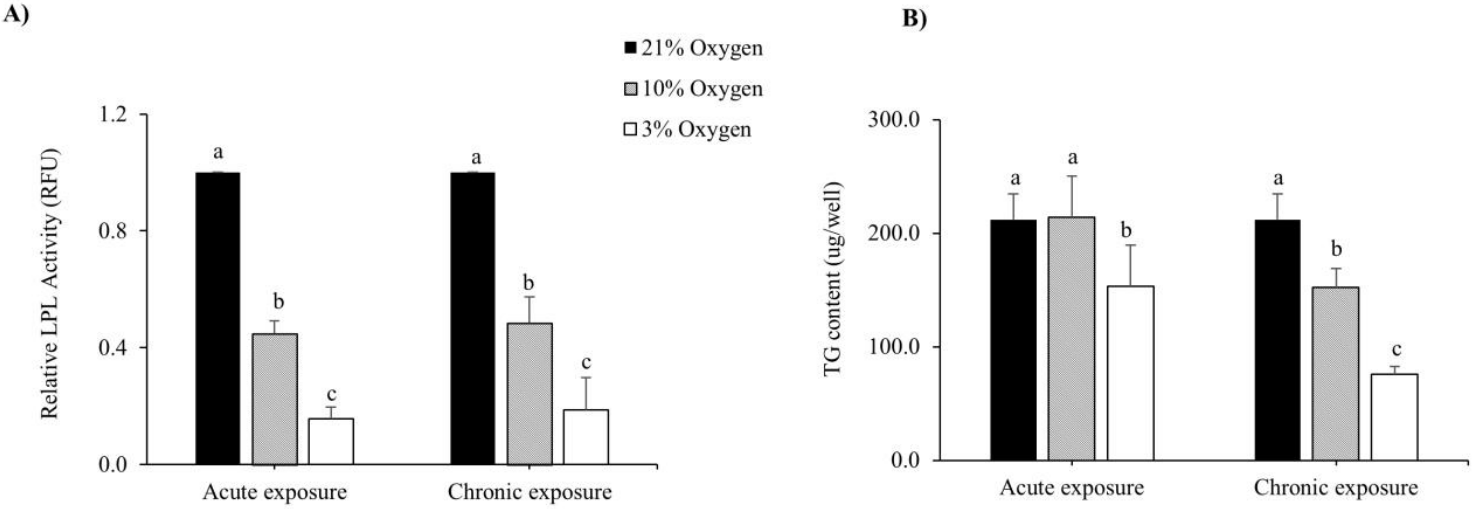

Figure 1. Effects of acute (t=24h, after differentiation) and chronic (t=14d, during differentiation) exposure to different oxygen tensions (21%, 10%, and 3%) on differentiated human preadipocytes **(A)** lipoprotein lipase (LPL) activity, and **(B)** triglycerides (TG) content. Results are from 3 independent experiments performed in triplicate. Values are mean ± standard deviation. Bars with different letters are statistically different.

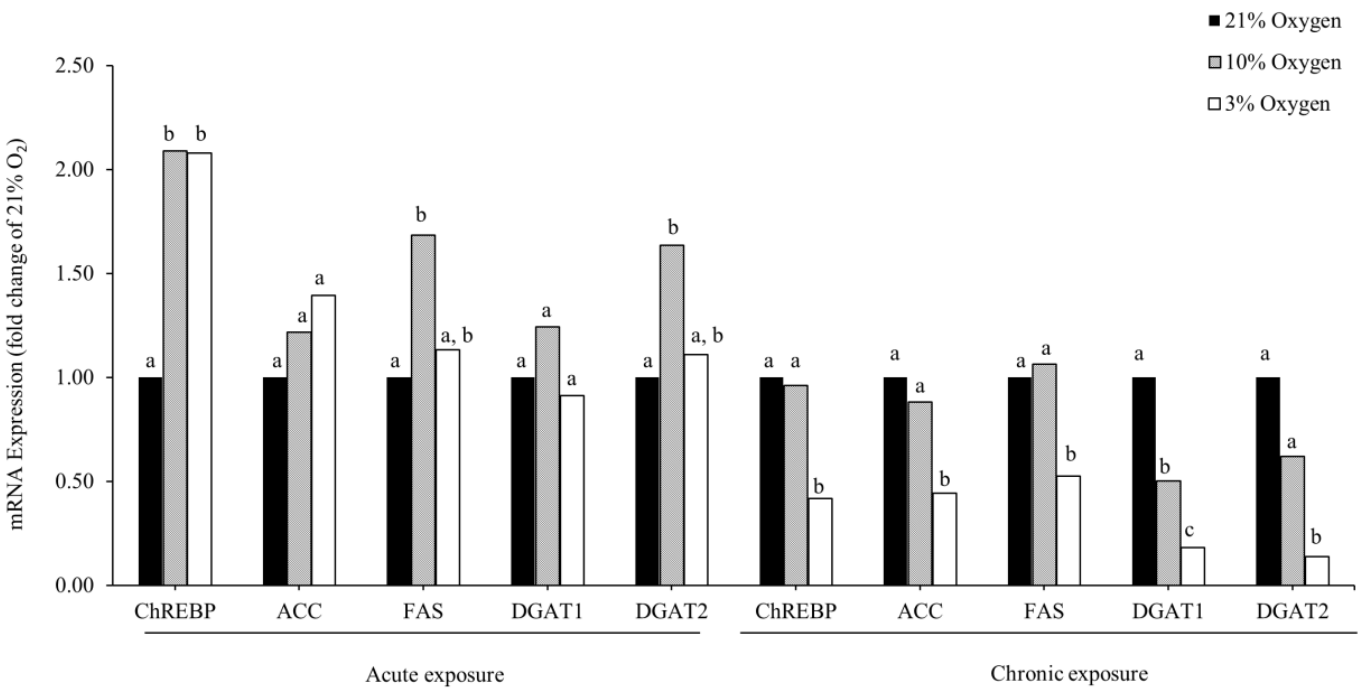

Figure 2. Effects of acute (t=24h, after differentiation) and chronic (t=14d, during differentiation) exposure to different oxygen tensions (21%, 10%, and 3%) on differentiated human preadipocytes carbohydrate response element-binding protein (ChREBP), acetyl-coA carboxylase (ACC), fatty acid synthase (FAS), diacylglycerol acyltransferase 1 (DGAT1), and diacylglycerol acyltransferase 2 (DGAT2) mRNA expression. Results are from 3 independent experiments performed in triplicate. Bars with different letters are statistically different.

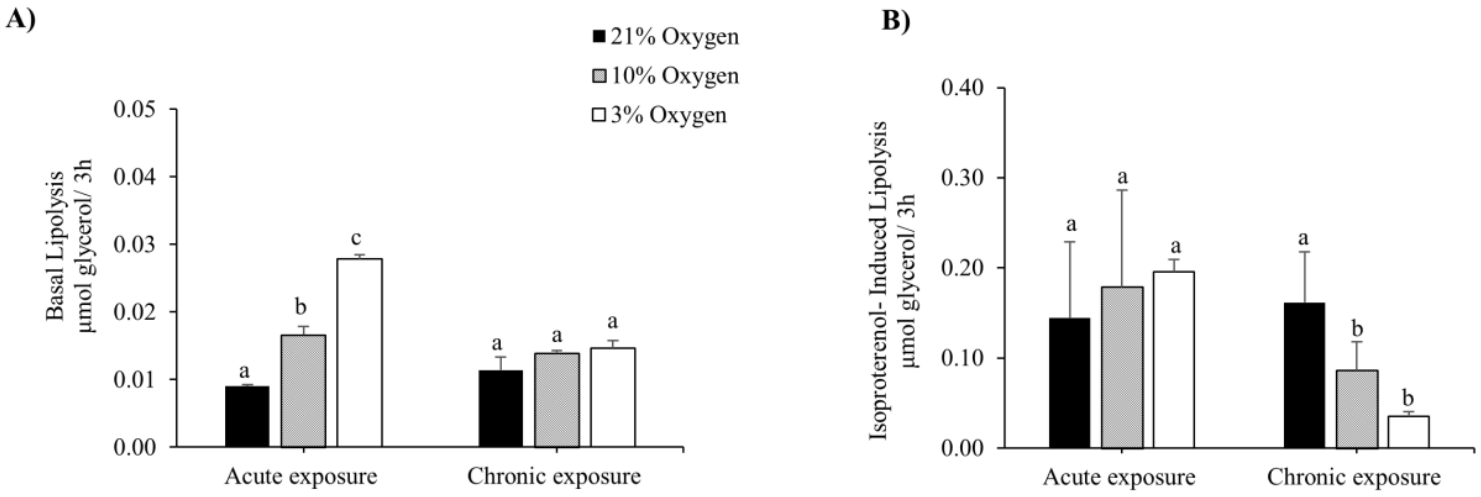

Figure 3. **(A)** Basal lipolytic rate as well as effect of **(B)** [10-5] M isoproterenol (β- adrenoceptors (AR) agonist), on differentiated human preadipocytes lipolysis using acute (t=24h, after differentiation) and chronic (t=14d, during differentiation) exposure to different oxygen tensions (21%, 10%, and 3%). Results are from 3 independent experiments performed in triplicate. Bars with different letters are statistically different.

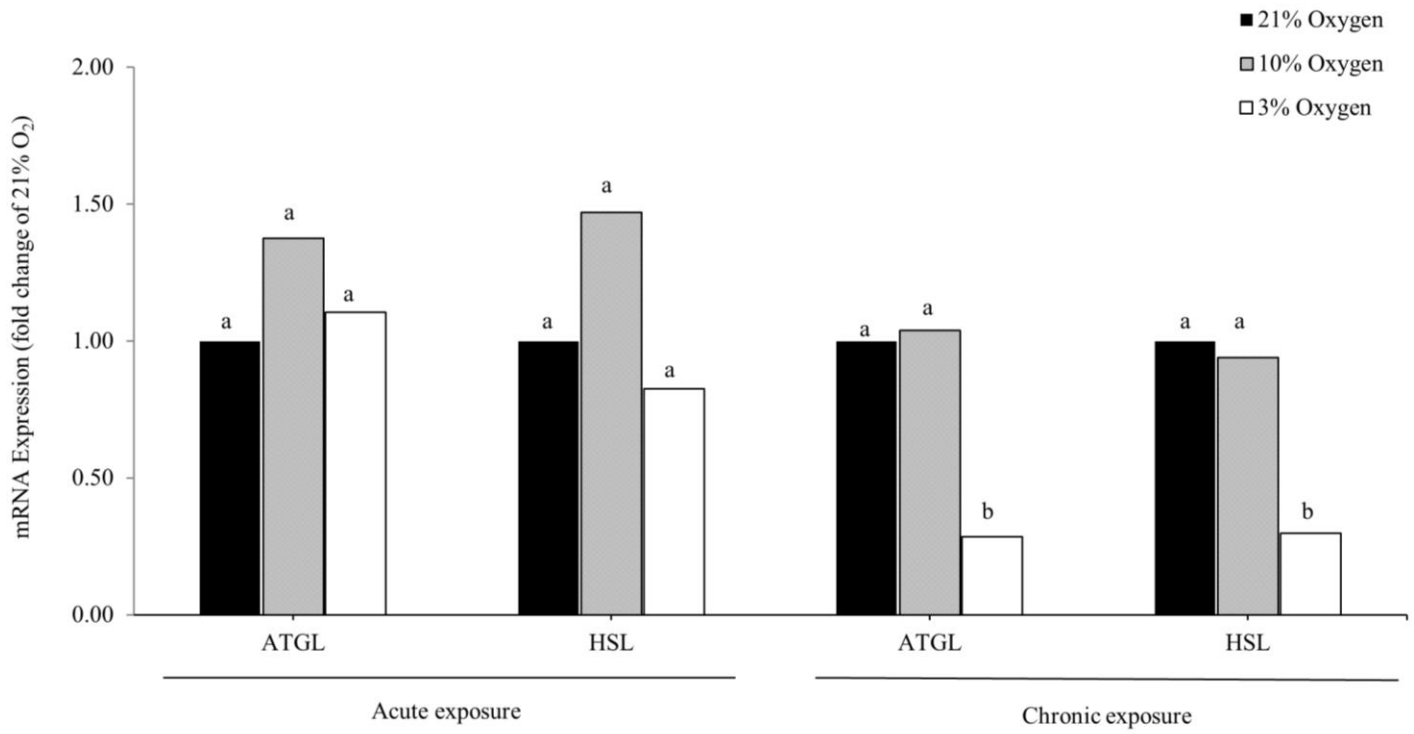

Figure 4. Effects of acute (t=24h, after differentiation) and chronic (t=14d, during differentiation) exposure to different oxygen tensions (21%, 10%, and 3%) on differentiated human preadipocytes adipose triglyceride lipase (ATGL), and hormone-sensitive lipase (HSL) mRNA expression. Results are from 3 independent experiments performed in triplicate. Bars with different letters are statistically different.

3.2 Thesis article #2: Effects of Acute Hypoxia on Human Adipose Tissue Lipoprotein Lipase Activity and Lipolysis

This article was accepted for publication on 29 June 2016 by the *Journal of Translational Medicine*, and has been formatted according to the thesis. The final published version can be found in Appendix B and permissions for publication can be found in Appendix C.

Effects of acute hypoxia on human adipose tissue lipoprotein lipase activity and lipolysis

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Abstract

Background: Adipose tissue regulates postprandial lipid metabolism by storing dietary fat through lipoprotein lipase-mediated hydrolysis of exogenous triglycerides, and by inhibiting delivery of endogenous non-esterified fatty acid to nonadipose tissues. Animal studies show that acute hypoxia, a model of obstructive sleep apnea, reduces adipose tissue lipoprotein lipase activity and increases non-esterified fatty acid release, adversely affecting postprandial lipemia. These observations remain to be tested in humans.

Methods: We used differentiated human preadipocytes exposed to acute hypoxia as well as adipose tissue biopsies obtained from 10 healthy men exposed for 6 h to either normoxia or intermittent hypoxia following an isocaloric high-fat meal.

Results: In differentiated preadipocytes, acute hypoxia induced a 6-fold reduction in lipoprotein lipase activity. In humans, the rise in postprandial triglyceride levels did not differ between normoxia and intermittent hypoxia. Non-esterified fatty acid levels were higher during intermittent hypoxia session. Intermittent hypoxia did not affect subcutaneous abdominal adipose tissue lipoprotein lipase activity. No differences were observed in lipolytic responses of isolated subcutaneous abdominal adipocytes between normoxia and intermittent hypoxia sessions.

Conclusion: Acute hypoxia strongly inhibits lipoprotein lipase activity in differentiated human preadipocytes. Acute intermittent hypoxia increases circulating plasma non-esterified fatty acid in young healthy men, but does not seem to affect postprandial triglyceride levels, nor subcutaneous abdominal adipose tissue lipoprotein lipase activity and adipocyte lipolysis.

Keywords: Intermittent hypoxia, Obstructive sleep apnea, Adipose tissue metabolism, Postprandial lipemia, Cardiovascular disease

Background

Obstructive sleep apnea (OSA) is a prevalent sleep disorder affecting approximately 5–15 % of middle-aged and older adults in the general population (Young et al. 2002). Individuals with OSA experience short periods of hypopnea, inducing intermittent hypoxia-hypercapnia/normoxia cycles. The most salient symptom of OSA is excessive daytime sleepiness, but its most important health consequence is an approximate two-fold increased risk of developing cardiovascular disease (CVD) such as coronary artery disease, heart failure, or stroke (Government of Canada 2010). The link between OSA and CVD could be explained by the fact that OSA may disturb lipid metabolism and lead to a deteriorated blood lipid profile. It has been shown that individuals with OSA display increased triglyceridemia (by ~30 %), independent of age and body mass index, compared to individuals without OSA (Newman et al. 2001).

Adipose tissue plays a central role in energy substrate homeostasis by acting as a crucial regulator of whole-body lipid flux. More specifically, in response to metabolic demand, triglyceride (TG) stored within adipocytes can be hydrolyzed into fatty acids and glycerol to be released for use by non-adipose organs. Postprandially, the transport of lipoprotein lipase (LPL) from intracellular vacuoles to the capillaries endothelium promotes the hydrolysis of dietary TG and subsequent uptake of dietary fatty acids within adipocytes (Coppack et al. 1990, Samra 2000). The proper regulation of lipid uptake and secretion by the adipose tissue is thought to be critical to limit ectopic fat storage in metabolically important tissues, namely the liver, skeletal muscles, and pancreatic beta cells, and to prevent chronic disorders such as type 2 diabetes and CVD (McGarry 1992, Lewis et al. 2002).

Recent animal studies demonstrated that chronic intermittent (Drager et al. 2012, Yao et al. 2013) and acute hypoxia (Jun et al. 2012) increase hepatic TG secretion in the fasted state and delay TG clearance in the postprandial state. These changes appear to be caused, in part, by (a) an increase in lipid influx to the liver due to an increase in adipose tissue lipolysis and by (b) a suppression of LPL activity by more than 50 %. While the increase in adipose tissue lipolysis has been linked to the increase in sympathetic drive observed during hypoxia, the reduction in adipose tissue LPL activity appears to be explained by the upregulation of an important post-translational repressor of LPL, angiopoietin-like protein 4 (ANGPTL4) (Drager et al. 2012).

Despite evidence from animal studies indicating that hypoxia considerably affects adipose tissue functions, blood lipid profile, and potentially the risk of CVD or type 2 diabetes in OSA patients, data regarding these effects in humans is crucially lacking. Therefore, the objective of this study was to investigate the effects of hypoxia on human adipose tissue LPL activity and adipocyte lipolysis. We hypothesize that: (1) In differentiated human preadipocytes, acute exposure to hypoxia inhibits LPL activity, and (2) In humans, acute intermittent hypoxia leads to an exaggerated elevation in postprandial TG concentrations consequent to an increase in adipocyte lipolysis and/or an impairment in subcutaneous abdominal adipose tissue LPL activity.

Methods

In vitro experiments

Culture of human preadipocytes

Cryopreserved subcutaneous abdominal preadipocytes from two Caucasian female (average age: 39 y; mean body mass index: 22.74 kg/m) were obtained from Zen-bio (NC, USA) and differentiated according to manufacturer's instructions ("Cell Manuals" n.d.). Briefly, preadipocytes were plated at a density of 4 × 10 cells/cm in 24-well plates, and proliferated in preadipocytes medium (PM-1) for 48 h, or until confluence was reached. Differentiation was induced by substituting the culture media for adipocyte differentiation medium (DM-2) in which cells were maintained for 7 days. Cells were then fed by replacing the culture medium with the adipocyte maintenance medium (AM-1), and maturation was continued for another week. Fourteen days post-induction, cells were transferred to basal medium (BM-1) and incubated in either hypoxic (3% oxygen) or normoxic (21 % oxygen) conditions (Wang et al. 2007), for 24 h. No cell lost was observed at the end of each treatment. After treatments, media were collected and cells were washed three times with phosphate buffer saline (PBS). To assess LPL activity, cells were incubated for 30 min in their respective oxygen conditions, in presence of BM-1 containing 100 U/ml heparin. BM-1/heparin media were collected, cells were wash three times with PBS and lysed with RLT buffer (QIAGEN) containing 10 % β-mercaptoethanol.

RNA isolation and RT-PCR

Total RNA was extracted from cell lysates using QIAGEN RNeasy Mini kits, following the manufacturer's instructions. Complementary DNA was prepared from 300 ng of total RNA using QIAGEN reverse transcriptase kit, following elimination of genomic DNA using QIAGEN gDNA WipeOut. Since there is no discrepancy between protein level and mRNA expression of Angiopoietin-like 4 (ANGPTL4), only the gene expression was determined (Drager et al. 2012). Gene expression was determined by real-time PCR using Eva Green Master Mix (Montreal Biotech) on a Rotor-Gene. Quantitect primers (forward and reverse) for ANGPTL4,

metallothionein-3 (MT3), and β-actin were purchased from QIAGEN, with β-actin serving as the reference gene. Delta-delta CT (cycle threshold) analyses were conducted using the Rotor-Gene 6000 software version 1.7.

LPL activity

LPL activity in differentiated preadipocytes was measured in 50 µl of BM-1-Heparin using the EnzChek Lipase Substrate (Thermo Fisher Scientific), a fluorescent triacylglycerol analog, at a final concentration of 0.62 µM in presence of 18-carbon zwittergent (0.0125 %), 0.15 M NaCl and 20 mM Tris–HCl pH 8. Fluorescence emission kinetics were followed over 1 h at 37 °C and fluorescence from blank wells was subtracted. Average blank-adjusted RFU (relative fluorescence units) are reported here. All samples from an identical experiment were assessed simultaneously, alongside positive controls containing bovine LPL. LPL activity in adipose tissue biopsies was determined similarly, excepted that LPL was first extracted from thawed subcutaneous abdominal adipose tissue samples by incubation at 28 °C for 40 min in Krebs–Ringer buffer containing 1 % BSA (bovine serum albumin) and 0.05 mg/ml heparin as previously described (Taskinen et al. 1980, Imbeault et al. 1999).

***In vivo* experiments**

Subjects

Ten healthy young men were recruited from the University of Ottawa population. Study subjects provided written consent and the study protocol was approved by the Research and Ethics Board of the University of Ottawa. Exclusion criteria included: history of physician-diagnosed asthma or other respiratory illness, hypertension, CVD, diabetes, habitual sleep duration of less than 7 h per night, habitual bed time occurring after midnight, shift work, and current smoking habit.

Anthropometric measurements

Body weight was determined with a standard beam scale (HR-100, BWB-800AS; Tanita, Arlington Heights, IL) and height was measured using a standard stadiometer (Perspective Enterprises, Portage, Michigan, USA). Waist circumference was measured following World Health Organization procedure. Percentage of fat mass (%FM), total fat mass (FM) and fat free mass (FFM) were measured using dual energy X-ray absorptiometry (DXA) (General Electric Lunar Prodigy, Madison, Wisconsin; software version 6.10.019). Resting energy expenditure (REE) was measured by indirect calorimetry using a Vmax Encore 29 System metabolic cart (VIASYS Healthcare Inc, Yorba Linda, CA).

Experimental protocol

This was a randomized crossover study consisting of two experimental sessions. Prior to each experimental session, volunteers were counseled to sleep at least 7 h per night, to restrain from any exercises and caffeine for at least 24 h, and to consume a provided standardized evening dinner between 7:00 and 8:00 PM (lasagna of 3220 kJ or 770 kcal; 42% from carbohydrates, 28% from fat, and 30% from protein). On study days, volunteers presented themselves at the laboratory at 7:30 AM after a 12-h overnight fast. Weight measurements were performed before an intravenous line was inserted in the antecubital vein for blood sampling and kept patent with a continuous infusion of 0.9% saline. A baseline subcutaneous abdominal adipose tissue biopsy (detailed below) was then performed. Volunteers were thereafter asked to consume a fat-rich liquid meal (59% of calories from fat, 28% from carbohydrates and 13% from protein) providing one-third of their estimated daily energy expenditure (obtained by indirect calorimetry during a preliminary session) times a physical activity factor of 1.375 (Harris and Benedict 1918), and

were then exposed to either intermittent hypoxia or to ambient air (normoxia) for 6 h. Volunteers remained in a semirecumbent position, and occupied themselves by watching television. Sleep was not allowed. Oxyhemoglobin saturation and heart rate were continuously monitored by pulsed oximetry. A second adipose tissue biopsy was performed 3 h after meal ingestion.

OSA simulation (intermittent hypoxia)

Subjects had to wear a well-fitted oro-nasal mask with a two-way Hans Rudolph non-rebreathing valve connected to an inspiratory line, as reported by Louis et al. (Louis and Punjabi 2009). During normoxia session, ambient air only was provided. During intermittent hypoxia sessions, pressurized medical N_2 was intermittently introduced in the inspiratory line. Oxyhemoglobin saturation (SpO_2) was allowed to drop to 85%, at which point the flow of N was stopped until the oxyhemoglobin saturation returned to the pre-exposure values (~98%). Intermittent hypoxia was well-tolerated and presented no adverse effects. This experimental setup allowed us to produce 17.3 ± 3.8 hypoxic events per hour, which is comparable to moderate OSA.

Fasting and postprandial plasma metabolic parameters

Plasma was obtained by centrifugation at 3000 rpm for 10 min at 4°C immediately after blood collection. Commercially available colorimetric enzymatic assays were used to measure plasma total triglyceride, glucose, non-esterified fatty acid (NEFA) (Wako Chemicals USA Inc, VA, USA) and lactate concentrations (Eton Bioscience Inc. NL, USA). Commercially available enzyme-linked immunosorbent assay kits were used to determine insulin (EMD Millipore, MA, USA) and catecholamines (Rocky Mountain Diagnostics Inc, CO, USA), as previously described (Imbeault et al. 2009).

Subcutaneous abdominal adipose tissue biopsy

On both experimental sessions, two subcutaneous abdominal fat biopsies were performed, one before and one 3 h after meal ingestion. Biopsies were performed in the periumbilical region (within 4–6 cm), as previously described (Taskinen et al. 1980). On the second experimental session, biopsies were performed 4 cm underneath the incisions made on the first session.

Adipocyte lipolysis

Immediately after the biopsy, roughly 100 mg of fresh adipose tissue, free of capillaries, were digested with collagenase (1 mg/ml) in 4 % BSA Krebs–Ringer buffer at 37 °C and filtered through a nylon mesh. Adipocytes were isolated by centrifugation (500 rpm for 2 min), and washed twice with BSA-Krebs–Ringer buffer. Adipocyte density was then adjusted to 500 adipocytes/50 µl. With constant stirring, 50 µl aliquots of adipocytes suspension were distributed in 1.5 ml Eppendorf tubes, and incubated at 37 °C for 2 h in BSA-Krebs– Ringer buffer under 95 % O in presence of isoproterenol (0.001, 0.01, 0.1, 1 and 10 µM), epinephrine (0.001, 0.01, 0.1, 1 and 10 µM) and UK 14304 (0.0001, 0.001, 0.01, 0.1 and 1 µM). Epinephrine and UK 14304 tubes also contained adenosine deaminase (ADA). Lipolytic rate was determined by glycerol quantification using bioluminescence, as described by Mauriege et al. (Mauriège et al. 1999). Adipocyte density (cells/50 µl) was determined by counting and averaging the number of adipocytes in five 50 µl samples collected throughout the distribution step. Results are presented as µmol of glycerol released by 1 × 10 adipocytes over 2 h. Adipocyte size was obtained by analysing 10× digital images of adipocytes loaded on a hemocytometer using the Infinity Capture and Analyse software (Lumenera Corporation, ON, Canada). Each average adipocyte diameter was computed from at least 150 random individual measurements.

Statistical Analysis

SPSS version 12 for windows was used for data analysis (SPSS Inc. Chicago, IL, USA). Repeated measures analyses of variance (ANOVA) were performed with condition and time as within subject's parameters. Alpha was set at 0.05.

Results

LPL Activity in differentiated Human Preadipocytes

In vitro, hypoxia induced a significant 6-fold reduction ($p < 0.001$) in LPL activity (Fig. 1 a). mRNA levels of ANGPTL4, a repressor of LPL activity, and MT3, a gene known to be highly induced by hypoxia, were significantly increased by 27-fold ($p < 0.001$) and 70-fold ($p < 0.001$) respectively following hypoxia (Fig. 1 b, c).

Subject Characteristics

Metabolic and anthropometric characteristics of the 10 healthy men are represented in Table 1. Participants reported a good quality of sleep, according to the Pittsburgh Sleep Index (3.83 ± 2.71) (Buysse et al. 1989). On average, participants reported 7.3 h of sleep during the night prior to the experimental sessions. The average time between each experimental session was 7.4 days, and participants' weight (± 0.35 kg) did not differ between experimental sessions.

Oxyhemoglobin Saturation and Heart Rate Responses to Intermittent Hypoxia

Table 2 displays the variations in heart rate and oxyhemoglobin saturation during normoxia and intermittent hypoxia sessions. During intermittent hypoxia, an average of 17.3 ± 3.8 hypoxic cycles was induced per hour. Heart rate was significantly increased during hypoxic exposure, reaching an average peak increase of ~20 bpm.

Plasma Metabolic Parameters

Postprandial plasma TG, glucose, lactate, insulin, and NEFA levels during normoxia and intermittent hypoxia sessions are depicted in Fig. 2. Postprandially, TG levels increased significantly (time effect, $p<0.001$) but did not differ between normoxia and intermittent hypoxia sessions (Fig. 2a). Regardless of time, glucose and lactate were significantly greater during intermittent hypoxia than normoxia (condition effect, $p<0.05$). Both variables evolved in a similar manner over time (time effect, $p<0.01$) (Fig. 2b, c).

After a peak at 30 min, insulin levels declined more steeply during intermittent hypoxia sessions (condition × time interaction, $p<0.05$) (Fig. 2d). Regardless of time, NEFA levels were significantly higher during intermittent hypoxia sessions (condition effect, $p<0.05$) (Fig. 2e). No difference in circulating epinephrine and norepinephrine concentrations were observed between experimental conditions (data not shown).

Subcutaneous Adipose Tissue Metabolism

Adipose tissue LPL activity (Fig. 3a) and ANGPTL4 expression (Fig. 3b) were affected neither by the meal nor the experimental conditions. Adipose tissue MT3 gene expression levels remain comparable before and after the meal in normoxia, but increased 4-fold under intermittent hypoxia. This interaction fell short of statistical significance (condition × time interaction, $p=0.1$) (Fig. 3c).

Basal and stimulated lipolytic rate assessed from isolated subcutaneous abdominal adipocytes before and 3 h after the meal are presented in Fig. 4. A trend toward lower basal lipolytic rate in the postprandial phase compared to baseline measurements was observed in both conditions (effect of time, $p=0.1$, Fig. 4a). Adenosine deaminase (ADA)-stimulated lipolysis was

significantly and similarly reduced postprandially compared to baseline in both conditions (effect of time, $p<0.05$) (data not shown). The dose-dependent lipolytic responses to isoproterenol (β-adrenoceptor [AR] agonist) were significantly and similarly reduced postprandially in both conditions (effect of concentration, $p<0.01$) (Fig. 4b). Neither the meal nor the conditions affected the antilipolytic effects of epinephrine (mixed α2/β-AR agonist) and UK- 14304 (α2-AR agonist) (effect of concentration, $p<0.001$) (Fig. 4c, d).

Discussion

Using differentiated human preadipocytes and subcutaneous abdominal adipose tissue biopsies from healthy individuals, we investigated the effects of acute hypoxia on adipose tissue lipid storage and/or mobilization functions. We show that 24 h of hypoxia significantly inhibits the activity of a key enzyme involved in adipose tissue TG deposition, LPL, in differentiated human preadipocytes. To explore whether the inhibitory effect of hypoxia on adipose tissue functions are noticeable in humans, young, healthy men were exposed for 6 hours to acute intermittent hypoxia, an experimental model that has been proposed to study the metabolic effects of OSA. Acute exposure to intermittent hypoxia was sufficient to alter postprandial NEFA levels, as well as glucose and insulin levels, but did not alter circulating triglycerides nor subcutaneous adipose tissue lipid storage and/or mobilization functions.

Effects of hypoxia on LPL activity in differentiated human preadipocytes

To our knowledge, this is the first study examining the effects of hypoxia on LPL activity in differentiated human subcutaneous abdominal preadipocytes. Our results show a 6-fold reduction in LPL after a 24 h-incubation in hypoxic conditions. Consistently, ANGPTL4, a major post-

translational regulator of LPL activity which inactivates LPL at the plasma membrane of adipocytes (Makoveichuk et al. 2013), was significantly increased after hypoxia, as previously reported by Wood et al. (Wood et al. 2011). These observations confirm that the potential for lipid uptake of differentiated human preadipocytes is sensitive to an acute decrease in oxygen availability. It also complements recent evidence indicating that hypoxia impedes expression level of genes involved in de novo lipogenesis in human visceral adipose tissue (García-Fuentes et al. 2015).

Metabolic (non-Lipid) effects of intermittent hypoxia in humans

In order to determine whether the reduction in LPL activity, observed in differentiated preadipocytes exposed to hypoxia, is translated in vivo, 10 young, healthy men were exposed to intermittent hypoxia in the postprandial state. Intermittent hypoxia was chosen over chronic hypoxia based on its similarity to sleep apnea, a disorder that is associated with an altered lipid profile (Newman et al. 2001, Trzepizur et al. 2013). A fat-rich meal was also given to our participants based on numerous animal studies suggesting that postprandial triglyceride clearance is impaired by hypoxia (Jun et al. 2012, Yao et al. 2013). Our experimental setup clearly induced a systemic response: besides oxyhemoglobin desaturation cycles (by design), heart rate sharply and systematically increased during hypoxic cycles, reflecting a hypoxia-induced increase in sympathetic tone. As compared to values observed in normoxia condition, glucose and lactate levels were significantly increased after 90 min of intermittent hypoxia exposure, likely reflecting a shift in energy substrate utilization. Any changes in energy substrate partitioning, however, were impossible to confirm by indirect calorimetry, due to the constant changes in inspired and expired gas mixture.

Effects of intermittent hypoxia on lipid and adipose tissue metabolism

No significant difference in postprandial triglyceridemia excursion was observed during intermittent hypoxia. Consistently, postprandial LPL activity, measured from adipose tissue biopsies, was not different between normoxia and intermittent hypoxia conditions. Despite a 4-fold increase in abdominal subcutaneous adipose tissue MT3 expression, which likely suggests that adipose tissue have been exposed to reduced partial pressure in oxygen, ANGPTL4 expression was not induced by the intermittent hypoxia session. The absence of changes in LPL activity and ANGPTL4 expression suggests that the clearance rate of TG by adipose tissue was likely not affected by intermittent hypoxia in our study sample. These results are not consistent with those from animal studies (mice) demonstrating that acute exposure to hypoxia (Jun et al. 2012) or chronic intermittent hypoxia (Drager et al. 2012, Yao et al. 2013) delays plasma TG clearance and decrease subcutaneous LPL activity in white adipose tissue following a meal. These discrepancies, if not species-related, may be explained by the severity of the hypoxic stress. While the current study was conducted with intermittent hypoxia at a rate of 17.3 ± 3.8 events/hour for 6 h, Drager et al. (Drager et al. 2012) conducted their animal studies with a frequency of 60 hypoxic events/hour and Jun et al. (Jun et al. 2012) used constant hypoxia for 6h.

The slight but statistically significant increase in plasma NEFA after 120 min of intermittent hypoxia is in line with several past observations of increased NEFA in animals exposed to hypoxic conditions (Jun et al. 2012). This is typically explained by an increase in sympathetic tone, which stimulates adipose tissue lipolysis (Jun et al. 2012). Results of lipolytic responses in isolated adipocytes from adipose tissue biopsies suggest, however, that if an increase in lipolysis rate occurred in vivo, it did not translate into an altered ex vivo response to lipolysis stimulating/inhibiting agents. Instead, it appears that the meal provided to our participants had a

clear inhibiting impact on the adipocyte lipolytic activity. To the best of our knowledge, this is the first study to report ex vivo lipolytic response in adipocytes before and after the consumption of a meal. Our observations clearly support a strong suppression of NEFA release by isolated adipocyte of lean individuals in the postprandial phase. It is important to note, however, that despite the clear postprandial inhibition of lipolysis, adipocytes were still responsive to epinephrine and isoproterenol. Accordingly, the elevated plasma NEFA levels observed during intermittent hypoxia could still come from an increase in sympathetic drive, which should have been less present in the normoxia session. Other contributing factors to the increase in plasma NEFA during the intermittent hypoxia session include an earlier relief of lipolysis inhibition by insulin, and/or a decrease in circulating fatty acid utilisation by peripheral organs, leading to their accumulation in circulation. An increase in NEFA levels, in the long term, could lead to an increase in concentration of very low-density lipoprotein, small dense low-density lipoprotein particles, and elevated apolipoprotein B concentrations in plasma, all of which are associated with increased risk of coronary heart disease and stroke (Carlsson et al. 2000).

Some limitations of this study warrant discussion. First, in our in vitro experiments, only two different oxygen concentrations were tested: 3 % and 21% O_2. Since it has been reported that adipocytes are sensitive to even relatively small changes in oxygen level within the physiological range (Wang et al. 2007, Trayhurn 2013) further studies with different concentrations of oxygen could be undertaken. Limitations of our in vivo studies includes: the duration of intermittent hypoxia, which was brief, and limited to only 6 h in order to limit burden and potential side-effects on the hypoxia naïve participants; the severity of the intermittent hypoxia, which was equivalent to moderate OSA; and the homogeneity of our study sample, which consisted exclusively of healthy young men (Government of Canada 2010). All these limitations limit the

generalisation of our metabolic observation to individuals suffering from OSA. OSA patients are likely exposed to intermittent hypoxia on a daily basis, and a large proportion of them exhibit metabolic complications (Drager et al. 2013)—increased adiposity, dyslipidemia, and insulin resistance (consequently of OSA or not)—that may synergistically exacerbate the negative lipid-altering effects of intermittent hypoxia. Finally, adipose tissue LPL activity is both sex and depot sensitive (Imbeault et al. 1999). One could argue that these confounding factors may explain part of the discrepancy between our in vitro and in vivo observations since preadipocytes were obtained from female donors while our in vivo experiments included only male subjects. While it is possible that sex and depot can affect adipocytes responses to hypoxia, it should be emphasized that our in vitro approach served only as a proof of concept that differentiated human fat cells, regardless of the donor's sex or adipose tissue depot, show a reduction in LPL activity under hypoxia. Regarding our choice of sampling site, the periumbilical region was chosen because the subcutaneous abdominal adipose tissue is responsible for most (45-50%) of the clearance of exogenous lipids in humans (McQuaid et al. 2011, Koutsari et al. 2011). The remaining of the postprandial triglyceride clearance is proposed to be LPL-mediated in various other sites such as the subcutaneous femoral and visceral adipose tissues as well as the heart. Future studies remain to be performed to investigate how these other sources of LPL activity could be affected by intermittent hypoxia and to examine whether intermittent hypoxia affects the various sources of LPL similarly in men and women.

Conclusions

Our in vitro results indicate that hypoxia significantly inhibits lipoprotein lipase activity in differentiated human preadipocytes, while in vivo observations show that an acute session of

intermittent hypoxia significantly increases postprandial NEFA levels, but not postprandial circulating TG, adipose tissue LPL activity, or adipocyte lipolysis, in healthy young men.

Author contributions

All authors had full access to all of the data in the study and gave final approval of the submitted version. Study design and conduct: PI, JFM, EC and BM. Data collection and analysis: JFM, EC, PI and BM. Data interpretation: PI, JFM, BM. Manuscript writing: BM, JFM and PI. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing financial interest.

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Table 1

Characteristics of the participants (n = 10 men)

Variable	**Mean ± standard deviation**
Age (y)	22.8 ± 2.8
Body weight (kg)	84.5 ± 9.8
Height (cm)	181.7 ± 4.7
Body Mass Index (kg/m^2)	25.6 ± 2.3
Waist circumference (cm)	84.9 ± 5.1
Fat mass (kg)	12.5 ± 4.5
Lean mass (kg)	69.4 ± 11.2
Body fat (%)	15.3 ± 4.1
Subcutaneous abdominal adipocyte diameter (µm)	72.8 ± 5.7

Table 2

Summary of heart rate and oxyhemoglobin saturation (SpO_2) during normoxia and intermittent hypoxia sessions

		Normoxia	**Intermittent Hypoxia**
	Exposure time (min)	360.0	350.5 ± 16.7
	Frequency/hour	0	17.3 ± 3.8
Heart rate (BPM)	Mean	67.8 ± 11.9	71.7 ± 11.6
	Maximum	116.0 ± 16.6	120.5 ± 9.2*
SpO_2 (%)	Mean	96.8 ± 1.3	90.2 ± 1.1*
	Maximum	98.1 ± 0.4	98.4 ± 0.5
	Minimum	93.2 ± 3.9	64.3 ± 5.9*
Time SpO_2 (minutes)	≤90%	0	124.1 ± 31.6
	≤85%	0	50.8 ± 14.5
	≤80%	0	25.8 ± 7.9

Datas are mean ± standard deviation. *statistical difference between normoxia and intermittent hypoxia ($p<0.05$).

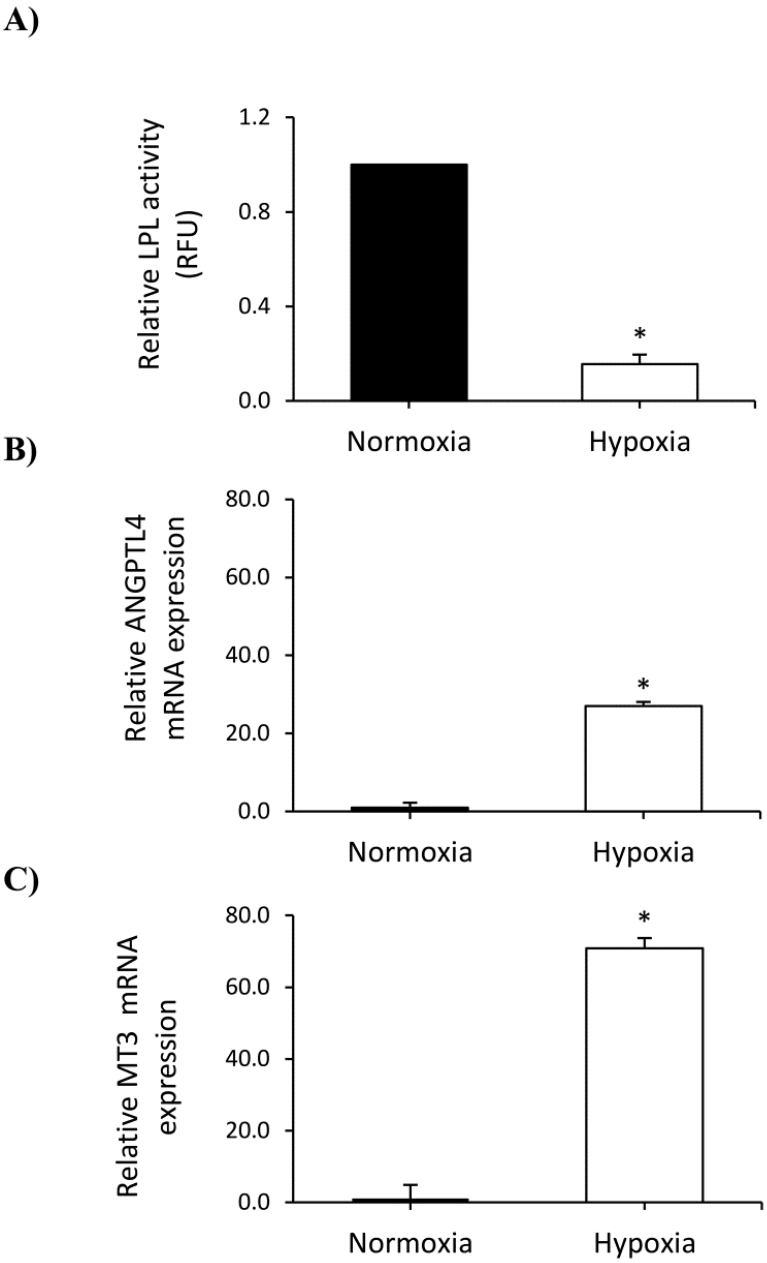

Fig. 1. Effect of normoxia (21 % oxygen) or hypoxia (3 % oxygen) on **a** lipopoprotein lipase activity, **b** Angiopoietin like 4 (ANGPTL4) gene expression and **c** metallothionein-3 (MT3) gene expression in differentiated human preadipocytes. Results are from 3 independent experiments performed in triplicate. Values are mean ± standard deviation. Significant difference between experimental sessions at *p < 0.001.

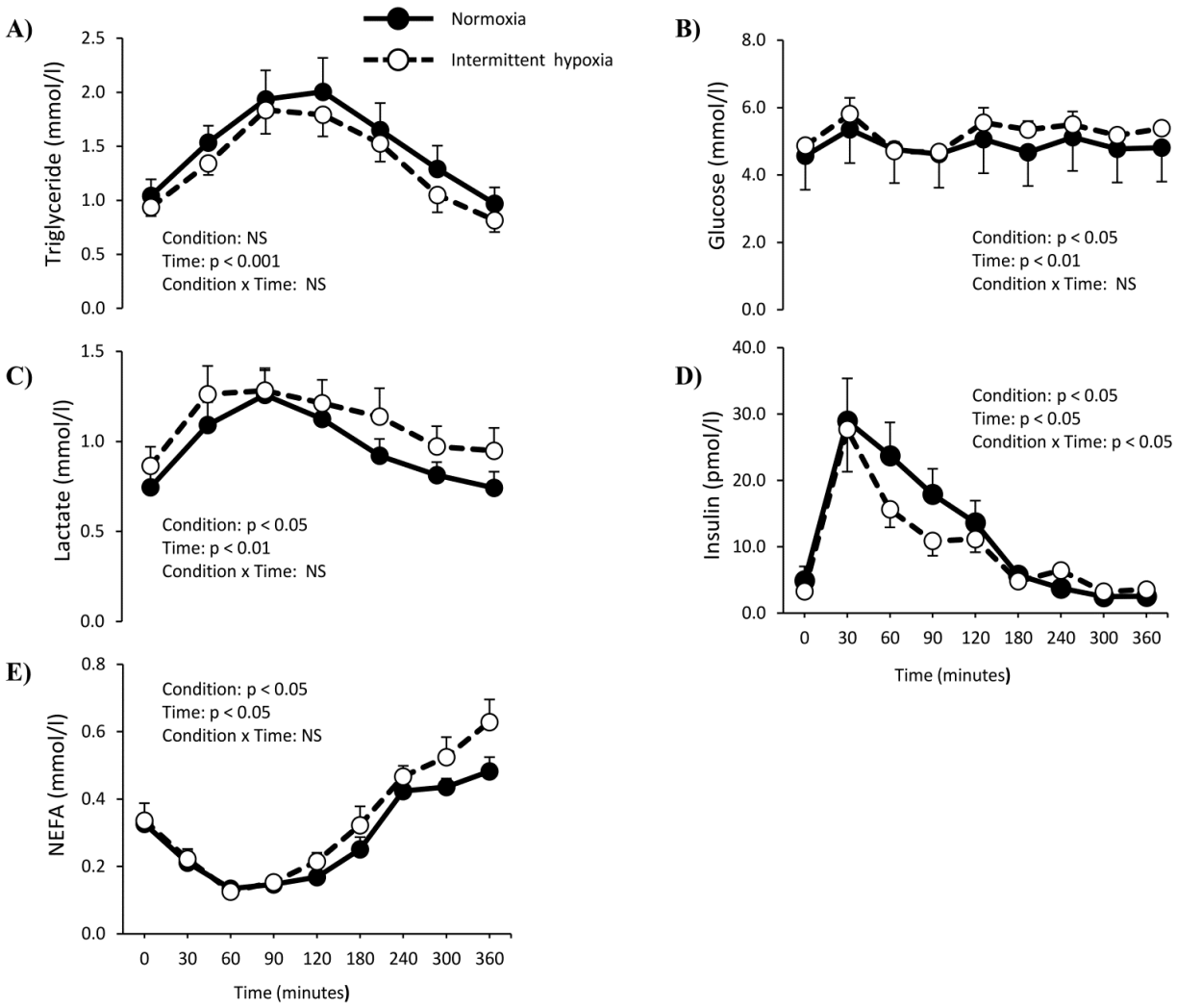

Fig. 2. Effect of normoxia or intermittent hypoxia on fasting and postprandial plasma **a** triglyceride, **b** glucose, **c** lactate, **d** insulin and **e** non-esterified fatty acids (NEFA) levels in healthy men. Values are mean ± standard error. NS not significant.

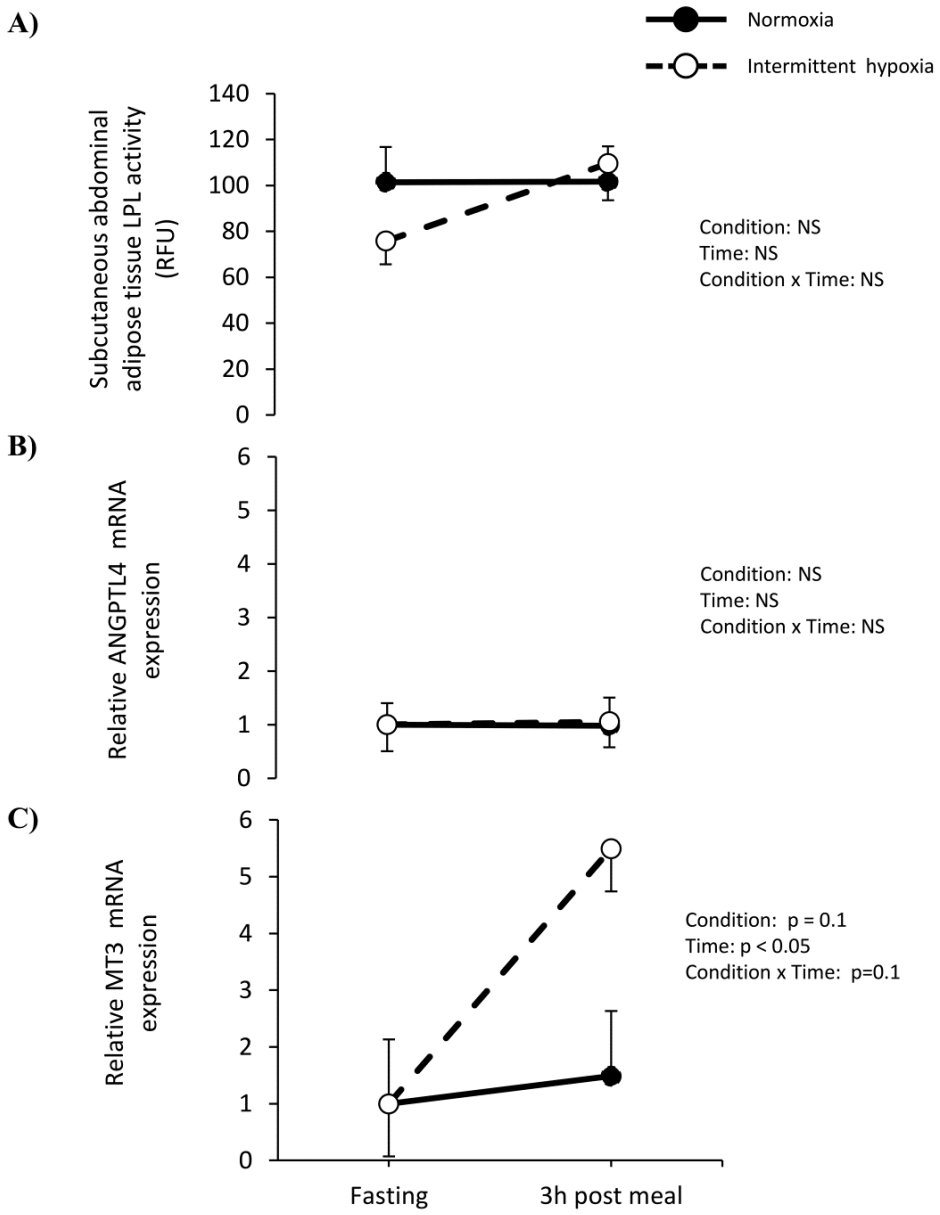

Fig. 3. Subcutaneous adipose tissue **a** lipoprotein lipase (LPL) activity, **b** angiopoietin-like 4 (ANGPTL4) gene expression and **c** metallothionein-3 (MT3) gene expression measured before (fasting) and 3 h post meal under normoxia and intermittent hypoxia in healthy men. Values are mean ± standard error. NS not significant.

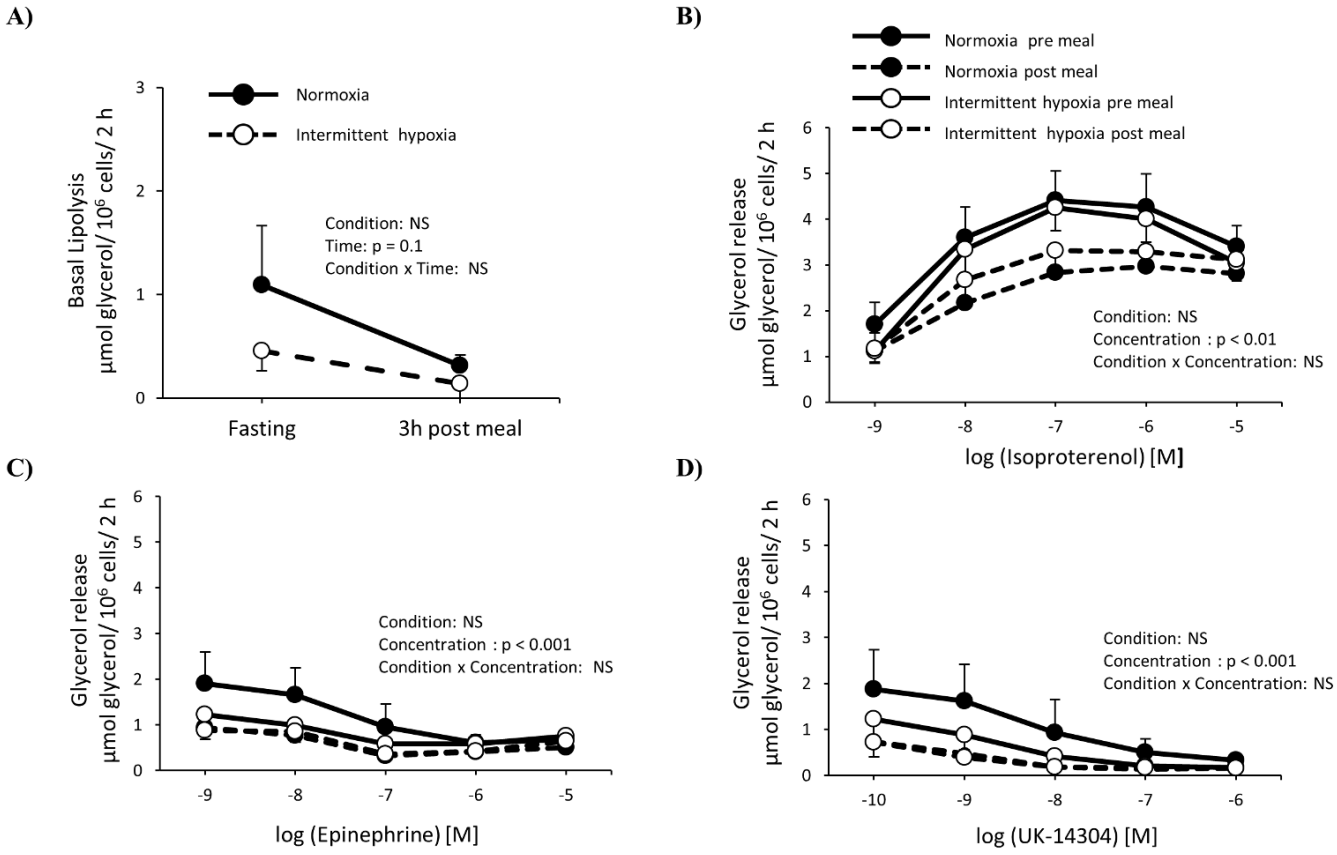

Fig. 4. **a** Basal lipolytic rate as well as effect of **b** isoproterenol (β- adrenoceptors (AR) agonist), **c** epinephrine (mixed α2/β-AR agonist) and **d** UK-14304 (α2- AR agonist) on lipolysis in subcutaneous abdominal isolated adipocytes of healthy men before and 3 h after a meal under normoxia and intermittent hypoxia. Values are mean ± standard error. NS not significant.

3.3 Thesis article #3: No Effect of Acute Normobaric Hypoxia on Plasma Triglyceride Levels in Fasting Healthy Men

This article is under revision to *Applied Physiology, Nutrition, and Metabolism*, and has been formatted to the thesis.

No Effect of Acute Normobaric Hypoxia on Plasma Triglyceride Levels in Fasting Healthy Men

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Abstract

Circulating fatty acids are a major systemic energy source in the fasting state as well as a determinant of hepatic triglyceride (TG)-rich very low-density lipoprotein (VLDL) production. Upon acute hypoxia, sympathetic arousal induces adipose tissue lipolysis, resulting in an increase in circulating non-esterified fatty acids (NEFA). Animal studies suggest that TG clearance may also be strongly reduced under hypoxia, though this effect has been shown to be dependent on temperature. Whether the hypoxia-induced rise in blood fatty acid concentrations effects fasting TG levels in humans under thermoneutral conditions has not been investigated. TG, NEFA and glycerol levels, were measured in fasted healthy young men (n=10) exposed for six hours to either normoxia (ambient air) or acute hypoxia (fraction of inspired oxygen (FIO_2) = 0.12) in a randomized, crossover design. Participants were casually clothed and rested in front of a fan in an environmental chamber maintained at 28 °C during each trial. Under hypoxia, a significantly greater increase in NEFA occurred (condition x time interaction, p=0.049) and glycerol levels tended to be higher (condition x time, p=0.104), suggesting an increase in adipose tissue lipolysis. However, plasma TG levels did not change over time and did not differ between the normoxia and hypoxia conditions. In conclusion, acute exposure to normobaric hypoxia under thermoneutral condition in men during fasting state increased lipolysis without affecting circulating TG.

Keywords: acute hypoxia, high altitude, plasma triglyceride, non-esterified fatty acids, fasting healthy men.

Résumé

Les acides gras circulants sont une source majeure d'énergie ainsi qu'un déterminant de la production hépatique de lipoprotéines de très faible densité (very low-density lipoprotein, VLDL) riches en triglycéride (TG). Lors d'une exposition aiguë à l'hypoxie, l'activation sympathique induit la lipolyse du tissu adipeux et l'augmentation des concentrations d'acides gras non estérifiés (NEFA) circulants. Les études animales suggèrent que le catabolisme des TG en circulation peut aussi être fortement réduit sous hypoxie, bien que cet effet n'ait été observé que lorsque les animaux sont exposés à des températures stimulant la thermogenèse. Aucune étude à date n'a été menée afin de déterminer si la hausse des concentrations sanguines d'acides gras induite par l'hypoxie effet la triglycéridémie à jeun chez l'humain dans des conditions près de la thermoneutralité. Les concentrations de TG, de NEFA et de glycérol ont été mesurées chez de jeunes hommes en santé et à jeun (n=10) exposés 6 heures à de l'air ambiant (fraction inspirée en oxygène (FIO_2) = 0.21, normoxie) ou à de l'air appauvri en oxygène (FIO_2 = 0.12, hypoxie) de façon randomisée et selon un devis chassé-croisé. Pendant chaque session expérimentale, les participants étaient au repos et vêtus normalement devant un ventilateur à l'intérieur d'une chambre environnementale maintenue à 28 °C. Lors de la session hypoxique, les concentrations plasmatiques de NEFA ont augmenté davantage en fonction du temps (interaction condition x temps, p=0.049) et les concentrations plasmatique de glycérol tendaient à augmenter (condition x temps, p=0.104). Toutefois, les concentrations plasmatiques de TG n'ont pas changé au cours du temps et ne différaient pas entre les conditions normoxie et hypoxie. Dans l'ensemble, une exposition aiguë à l'hypoxie normobarique en condition thermoneutre chez des hommes en santé en situation de jeûne augmenté de façon la lipolyse du tissu adipeux sans affecter les TG circulants.

Mots-clés: hypoxie aiguë, haute altitude, triglycéride plasmatiques, acides gras non estérifiés, hommes en santé à jeun.

Introduction

Restriction in oxygen (O_2) supply and/or increased O_2 consumption can lead to oxyhemoglobin desaturation and tissular hypoxia (Brahimi-Horn and Pouysségur 2007, Johnson et al. 2010). Recent animal studies demonstrated that chronic (Drager et al. 2012, Yao et al. 2013) and acute exposure to hypoxia (Jun et al. 2012, 2013) induces large augmentations in circulating triglyceride (TG) by increasing hepatic TG secretion in the fasted state and delaying TG clearance in the postprandial state. Proper TG metabolism is critical for global energy homeostasis. Furthermore, it is thought that impaired lipid storage and over exposition of organs to circulating lipids can lead to ectopic fat storage and lipotoxicity, which have been linked to impaired insulin secretion and reduced peripheral insulin signaling as well as the development of chronic diseases such as type 2 diabetes and cardiovascular disease (CVD) (Kalofoutis et al. 2007, Miller et al. 2011).

In humans, some studies examined blood TG concentrations following exposure to different hypoxia environments (normobaric vs hypobaric, altitude from 2000m up to 8800m) for various durations (from 2 hours up to 8 months) (Whitten and Janoski 1969, Férézou et al. 1988, Young et al. 1989, Leaf and Kleinman 1996, Siqués et al. 2007, Stöwhas et al. 2013). Results from these studies are conflicting, with reported increases (Whitten and Janoski 1969, Young et al. 1989, Siqués et al. 2007), decreases (Férézou et al. 1988, Stöwhas et al. 2013) or no change (Leaf and Kleinman 1996) in fasting plasma TG concentrations with hypoxia. This lack of consistency regarding the effects of hypoxia on TG concentrations in humans may be a consequence of poor control for confounding factors such as physical activity and diet. Based on recent animal studies, another important confounding factor that may have not been properly controlled for is thermal conditions. Indeed, the effects of acute hypoxia on plasma TG concentrations have been

reported to be temperature-dependent and virtually absent in animals studied at thermoneutrality (Jun et al. 2013).

Fasting circulating TG concentrations reflect the balance between hepatic very low-density lipoprotein (VLDL)-TG secretion and peripheral VLDL-TG clearance (Parks et al. 1999, Barrows and Parks 2006). Hepatic VLDL-TG production, on the one hand, is thought to be a function of fatty acids availability for hepatic TG synthesis. In the fasting state, 70-80% of total liver VLDL-TG production derives from non-esterified fatty acids (NEFA) (Barrows and Parks 2006). NEFA availability, in turn, depends mainly on white adipose tissue lipolysis which is under both sympathetic and hormonal control (Desvergne et al. 2006) with catecholamines and insulin being respectively the main activator and inhibitor. The peripheral clearance of VLDL-TG, on the other hand, is catalyzed mainly by the lipoprotein lipase (LPL) and the hepatic triglyceride lipase (HL), the activity of both being assessable in post-heparin plasma (Després et al. 1999). Interestingly, we recently showed that hypoxia strongly reduces LPL activity in differentiated human preadipocytes (Mahat et al. 2016), but no study yet reported fasting post-heparin lipase activity in response to hypoxia in humans.

Despite relatively strong evidence from animal studies supporting an important deleterious impact of acute hypoxia on triglyceridemia (Jun et al. 2012, 2013), the effect of acute hypoxia on blood lipid homeostasis in fasting humans remains elusive. Therefore, the present study examined the effects of an acute 6-hour hypoxia exposure on plasma TG concentrations in resting and fasting healthy young men. Confounding factors such as physical activity and diet prior to the study, as well as ambient temperature during hypoxia were controlled. The fasting state was chosen to favor peripheral lipolysis and hepatic NEFA delivery and experimental sessions were conducted at thermoneutrality because humans, through proper clothing, usually

live in such conditions. Beside plasma lipid levels, proxies of adipose tissue lipolysis and plasma lipase activity were also investigated. We hypothesized that acute hypoxia exposure in the fasting state would increase plasma TG concentrations by increasing hepatic NEFA availability for VLDL-TG production and by decreasing peripheral TG clearance.

Materials and Methods

Subjects

Thirteen healthy young men (age: 18-39 y) were recruited from the University of Ottawa population. Two participants dropped out after completing one session due to schedule conflicts and 1 participant dropped out due to altitude sickness (headache and severe vomiting during exposure to hypoxia). Body mass and height were measured using a standard beam scale (HR-100, BWB-800AS; Tanita, Arlington Heights, IL), and a standard stadiometer (Perspective Enterprises, Portage, Michigan, USA). Body fat was estimated by dual energy X-ray absorptiometry (General Electric Lunar Prodigy, Madison, Wisconsin; software version 6.10.019). On average, subjects were 26 ± 5.6 years, 177.9 ± 4.6 cm tall and weighed 79.9 ± 8.8 kg of which 22.6 ± 10.7% was fat tissue. The average time between each experimental session was 6.4 days, and participants' weight (± 0.25 kg) did not differ between experimental sessions. Exclusion criteria included: a history of physician-diagnosed asthma or other respiratory illness, hypertension, CVD, diabetes, habitual bedtime occurring after midnight, shift work, and a current smoking habit. Study subjects provided written consent and the study protocol was approved by the Research and Ethics Board of the University of Ottawa.

Experimental Protocol

This was a randomized crossover study consisting of two experimental sessions. Prior to each session, volunteers were counseled to sleep at least 7 hours per night, refrain from any exercise, caffeine and alcohol for at least 36 hours, and to consume the same evening dinner the day before each session. Participants wear their usual interior cloths. During each trial, an 18" diameter mechanical fan (High 117 velocity orbital air circulator, Whirlpool, Benton Harbor, MI, USA) set at an appropriate speed (maximum air velocity of ~4.0 m/s) was used to ensure the participants thermal comfort. The temperature and relative humidity were stable at 28 °C and 45% respectively during the experimental sessions. Participants were only allowed to drink water. Before each session, a catheter was inserted in the antecubital vein for blood sampling. The line was flushed with 10 ml of physiological saline after each blood draw to prevent coagulation and keep the catheter patent. Three milliliters of blood were discarded before each draw to remove the saline from the sampling line and prevent any dilution of the blood sample. Blood samples were collected in tubes containing ethylenediaminetetraacetic acid (EDTA). Volunteers were exposed to hypoxia (fraction of inspired oxygen (FIO_2) = 0.12) and to ambient air (normoxia) for 6 hours on 2 different sessions in a randomized cross-over fashion. Volunteers remained in a semi-recumbent position, and occupied themselves by watching television. Sleep was not allowed. Oxyhemoglobin saturation and heart rates were continuously monitored by pulsed oximetry using a Masimo, Radical 7 unit (Masimo, Irvine, CA, USA). Blood pressure was measured upon arrival, at mid experiment (T180) and finally at the end of the experimental session (T360) with an automatic sphygmomanometer (American Diagnostic Corporation, E-sphyg 2, Hauppage NY, USA) following the Canadian Society of Exercise Physiology (CSEP) standard procedures ("CSEP-PATH: Physical Activity Training for Health" n.d.).

Normobaric Hypoxia Exposure and Altitude Sickness Symptoms

All sessions were performed in an environmental chamber at the University of Ottawa. During the normoxia sessions, only ambient air was used (FIO_2 = 0.21). During hypoxia, O_2 extractors (CAT 12, Altitude Control Technologies, Lafayette, Colorado, USA) connected to the environmental chamber kept FIO_2 level stable at 12%. The CAT system uses 2 stable zirconium O_2 sensors in parallel to detect random sensors drift. The sensors are calibrated with ambient air (assuming an ambient air O_2 concentration of 20.94%) when sensors disagree by more than 0.5% O_2. During hypoxia, O_2 concentration was also continuously monitored by the constantly self-calibrating Vmax system used for indirect calorimetry. O_2 readings from both systems were always within 0.5%. No validated scale or questionnaire was used to monitor altitude sickness symptoms. Participants were instead frequently asked to report any discomfort related to altitude sickness with special attention to symptoms listed in the Lake Louise consensus scoring system (LLS): headache, gastrointestinal upset (anorexia, nausea, or vomiting), fatigue or weakness, and dizziness/light-headedness (Savourey et al. 1995).

Substrate Oxidation Rate

Substrates oxidation rates were determined by indirect calorimetry using a continuously self-calibrating Vmax Encore 29 System metabolic cart (VIASYS Healthcare Inc, Yorba Linda, CA). $\dot{V}O_2$ and $\dot{V}CO_2$ were measured for 30 minutes every hour and are expressed in STPD. $\dot{V}O_2$ and $\dot{V}CO_2$ were corrected to account for protein oxidation assuming a constant oxidation rate of 60 mg of protein per minute. Total carbohydrate (CHO), and lipid oxidation rates (g/min), were calculated using the protein-corrected $\dot{V}O_2$ and $\dot{V}CO_2$ with the following formulas (Elia 1991):

CHO oxidation rate (g / min) = 4.59 VCO_2 (l / min) – 3.23 VO_2 (l/min)

Lipid oxidation rate (g/min) = -1.70 VCO_2 (l / min) + 1.70 VO_2 (l/min)

Total energy expenditure was calculated using the estimated oxidation rates of CHO, lipids and proteins and the following energy equivalent: 3.896 kcal/g CHO, 9.751 kcal/g lipids, 4.708 kcal/g proteins.

Fasting Plasma Metabolic Parameters

Plasma was obtained by centrifugation at 3200 rpm for 12 minutes at 4 °C immediately after blood collection. Commercially available colorimetric enzymatic assays were used to measure plasma total TG, NEFA, glucose (Wako Chemicals USA Inc, VA, USA), lactate and glycerol (Cayman Chemical, Ann Arbor, Michigan). Insulin was measured by enzyme-linked immunosorbent assay (EMD Millipore, Darmstadt, Germany) as previously described (Imbeault et al. 2009, Mahat et al. 2016). Assay analyses were completed in duplicate and the intra-assay coefficients of variation were approximately 3%. Plasmatic lipolytic activity was measured using the Enzchek fluorescent TG-analog substrate (Basu et al. 2011) on blood samples collected 20 minutes following the injection of heparin (60 U/kg).

Statistical Analysis

All values in texts and figures are reported as mean ± standard deviation. SPSS version 12 for Windows was used for data analysis (SPSS Inc. Chicago, IL, USA). Repeated measure analyses of variance (ANOVA) were performed with condition and time as within-subject's parameters. A level of significance of $p<0.05$ was considered statistically significant.

Results

Side-Effects, Oxyhemoglobin Saturation and Heart Rate Responses to Acute Hypoxia

Fasting and hypoxia were well tolerated although most participants reported drowsiness. Only 1 participant experienced severe nausea and vomiting, leading to his exclusion of the study. One participant experienced severe dizziness while standing up and headache at rest and another participant experienced dizziness. Mean heart rate was significantly increased by approximately 20% in hypoxia (p=0.001) compared to normoxia. Mean oxyhemoglobin saturation was significantly reduced by more than 15% (p=0.001) during acute hypoxia compared to normoxia. Neither systolic blood pressure nor diastolic blood pressure differed between conditions.

Substrate Oxidation Rate

CHO and lipid oxidation rates during normoxia and hypoxia are depicted in **Figure 1**. CHO oxidation rate decreased significantly and similarly over time in both conditions (time effect, p=0.004) (**Figure 1A**). Lipid oxidation rate increased significantly and similarly over time in both conditions (time effect, p=0.003) (**Figure 1B**). Energy expenditure remained relatively stable over time and did not differ between experimental conditions (condition x time, p=0.609) (data not shown).

Plasma Metabolic Parameters

Fasting plasma TG, NEFA, glycerol, and insulin concentrations during normoxia and hypoxia are depicted in **Figure 2**. Plasma TG concentrations did not change over time and did not differ between experimental conditions (condition x time, p=0.544) (**Figure 2A**). The over-time increase in plasma NEFA concentrations was 95% greater under hypoxia (condition x time interaction, p=0.049) (**Figure 2B**). Glycerol concentrations remained constant under normoxia but tended to rise under hypoxia (condition x time, p=0.104) (**Figure 2C**). No differences were observed in total post-heparin plasma lipolytic activity between normoxia and hypoxia (p=0.233)

(data not shown). In both conditions, insulin levels significantly decreased over time (time effect, p=0.032) but tended to be higher overall during hypoxia (condition effect, p=0.061) (**Figure 2D**). Plasma glucose did not change over time and did not differ between experimental conditions (condition x time, p=0.461) (data not shown). Lactate levels remained relatively stable over time and were significantly higher during hypoxia (condition effect, p=0.028) (data not shown).

Discussion

This study aimed at determining the effect of an acute 6-hour bout of hypoxia on plasma TG concentrations in fasting healthy young males. Plasma TG are an important risk factor in the development of chronic diseases such as type 2 diabetes and cardiovascular diseases (Kalofoutis et al. 2007, Miller et al. 2011). Recent evidence from animal studies suggest that O_2 deprivation as experienced during journeys at altitude or in the context of diseases such as chronic obstructive pulmonary disease and sleep apnea, can substantially raise plasma TG concentrations (Drager et al. 2012, Jun et al. 2012, 2013, Yao et al. 2013). If such a response occurs in humans, individuals frequently exposed to hypoxia could be vulnerable to cardiometabolic complications. Some studies have reported conflicting results regarding the effect of hypoxia on plasma TG concentrations in humans (Whitten and Janoski 1969, Férézou et al. 1988, Young et al. 1989, Leaf and Kleinman 1996, Siqués et al. 2007), which could be due to a poor level of control for confounding factors such as physical activity, diet and environmental conditions, namely temperature. To our knowledge, this is the first well-controlled study to report the effects of acute normobaric hypoxia on fasting blood lipid profile in humans. We hypothesized that the combined effects of fasting (low insulinemia) and hypoxia (sympathetic arousal) would increase

NEFA delivery to the liver and increase plasma TG concentrations. We show that acute hypoxia progressively increases fasting NEFA (95% greater increase) and glycerol (33% increase) levels, suggesting an increased in adipose tissue lipolysis, but do not alter post-heparin plasma lipolytic activity nor circulating TG concentrations in young men with normal adiposity level.

Our findings corroborate observations by Leaf and Kleinman (Leaf and Kleinman 1996) who reported no change in plasma TG levels in humans exposed to simulated altitude, although they used less severe hypoxia conditions for a significantly shorter duration (FiO_2 = 16%, equivalent to 2200m altitude for 2 hours). Altogether, these observations seem conflicting with emerging evidence from animal studies showing a strong and rapid deleterious impact of hypoxia on lipid metabolism (Muratsubaki et al. 2003, Jun et al. 2012, 2013). Discrepancies in TG response to hypoxia may be related to two important factors, namely the thermal conditions and the nutritional status during which hypoxia occurs. In terms of thermal conditions, Jun et al. (Jun et al. 2013) have shown that, in mice, elevations in TG levels in response to hypoxia occurs in cold conditions (22 °C) but not at thermoneutrality (30 °C). They showed that cold up-regulates TG uptake in several tissues, namely brown adipose tissue, favoring sustained low TG levels in cold exposed rodents. At thermoneutrality, they demonstrate that mice TG levels are considerably higher than those of counterparts kept at 22 °C and that hypoxia no further increased plasma TG in these conditions. Whether a similar cold-hypoxia interaction is species-specific or occurs also in humans is unknown and warrant further research. However, recent experiments done on cold-acclimated humans showed no effect of a 5-hour cold exposure both on postprandial TG levels and dietary TG clearance rate (Blondin et al. 2017), suggesting that the lipid response to cold exposure is not as strong in humans as in rodents.

Regarding the influence of the nutritional status on the lipid response to hypoxia, experiments conducted in rodents by Muratsubaki et al. (Muratsubaki et al. 2003) showed that fasted rats, contrary to sated rats, show no increase in plasma TG levels when exposed for 5h to hypoxia (9.45% O_2). Fasting is recognized to decrease circulating TG concentrations by stimulating skeletal muscle LPL activity (Lithell et al. 1978) and whole-body fatty acid oxidation rates (Koutsari et al. 2011). Our observations suggest that the TG-lowering effects of fasting are not significantly altered acutely by hypoxia. To determine whether the nutritional status affects the lipid response to hypoxia in humans, a study examining the effect of hypoxia on lipid metabolism in the constantly fed state is currently being undertaken in our laboratory.

Despite no changes in plasma TG concentrations, the increase in plasma NEFA concentrations from baseline to 360 minutes was 95% greater under hypoxia compared to normoxia (**Figure 2**). It is worth noting that NEFA concentrations showed no evidence of stabilization after 6 hours, which suggest that higher plasma NEFA concentrations could be reached given a longer exposure. Exposure to reduced partial pressure of O_2 is well recognized to increase sympathetic activation (Hansen and Sander 2003, Prabhakar and Kumar 2010), which is an important activator of adipose tissue lipolysis. Consistently, the 24% increase in heart rate and the 33% increase in glycerolemia after 360 minutes of hypoxia exposure (**Figure 2**) are strong indicators that our experimental hypoxia exposure induced sympathetic arousal and stimulated lipolysis. Sympathetic activation is also well recognized to impair insulin sensitivity (Lambert et al. 2015). In this regard, Peltonen et al. (Peltonen et al. 2012) have elegantly demonstrated that the sympathetic nervous system activation induced by hypoxia disrupts insulin sensitivity in humans. Consistent with this observation, fasting insulin levels in our study were 66% greater after 360 minutes of hypoxia exposure (**Figure 2**) despite similar glucose levels (data not shown). This

apparent reduction in global insulin sensitivity, if present at the adipose tissue level, may also have contributed to a hypoxia-induced increase in lipolysis by lifting the inhibitory effect of insulin. Importantly, the major increase in plasma NEFA observed in the present study had no apparent effects on fatty acid oxidation according to indirect calorimetry measurements (**Figure 1**), which is concordant with previous studies suggesting that acute exposure to hypoxia has no significant effects of lipid oxidation rate (Brooks et al. 1991, Roberts et al. 1996). Since the oxidative disposal of fatty acids was seemingly not altered by hypoxia, it remains possible that the more abundant circulating NEFA under acute hypoxia exposure could eventually serve for hepatic VLDL synthesis. Nonetheless, we observed no significant changes in plasma TG concentrations. It appears unlikely that the higher insulinemia under hypoxia may have inhibited VLDL-TG secretion. Indeed, the suppressing effect of insulin on VLDL production has only been demonstrated under hyperinsulinemic conditions (Lewis and Steiner 1996) whereas in the present study, while insulin concentrations were higher under hypoxia after 6-hours, values were still below baseline (fasting) levels. Another possible explanation for the absence of shift in plasma TG despite an increase in NEFA availability is that NEFA are not utilized directly as an energy substrate in organs or for VLDL assembly in the liver, but first enter a temporary and probably expendable intracellular TG pool (Gibbons and Burnham 1991). This buffering capacity of the liver and/or peripheral organs could delay an increase in hepatic TG output in response to a rise in plasma NEFA and mitigate the effects of acute hypoxia on TG metabolism in humans. On the other hand, one could speculate that if tissue TG accumulation is not, in the longer term, compensated by an increase output in hepatic VLDL-TG or an increase in lipid oxidation, hypoxia could lead to ectopic fat storage and favor the development of metabolic abnormalities such as insulin resistance.

The present study has some limitations. First, the main endpoint, plasma TG concentrations, does not provide all the information regarding TG metabolism. The use of stably-labelled tracer infusions (Adiels et al. 2015) could allow to better estimate lipid and lipoprotein production and clearance rates and provide a more detailed picture of the effect of hypoxia on blood lipid homeostasis. Second, the duration of the hypoxia exposure was restrained to 6 hours to limit the burden, fasting time and potential side-effects on the hypoxia naïve participants. Whether a prolonged exposure could induce significant changes in plasma TG concentrations remains to be tested. A third limitation regards the homogeneity of our study sample, which consisted exclusively of healthy young men. This prevents the generalisation of our observations to women and/or metabolically deteriorated individuals. Whether individuals characterized by greater body fat % or by an adversely altered lipid metabolism could be affected differently by hypoxia will have to be addressed.

Conclusions

The current study supports the hypothesis that acute exposure to normobaric hypoxia increases adipose tissue lipolysis but the resulting increase in fatty acid availability does not translate into elevated circulating TG concentrations in fasting healthy men.

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Competing Interests

The authors declare that they have no competing financial interest.

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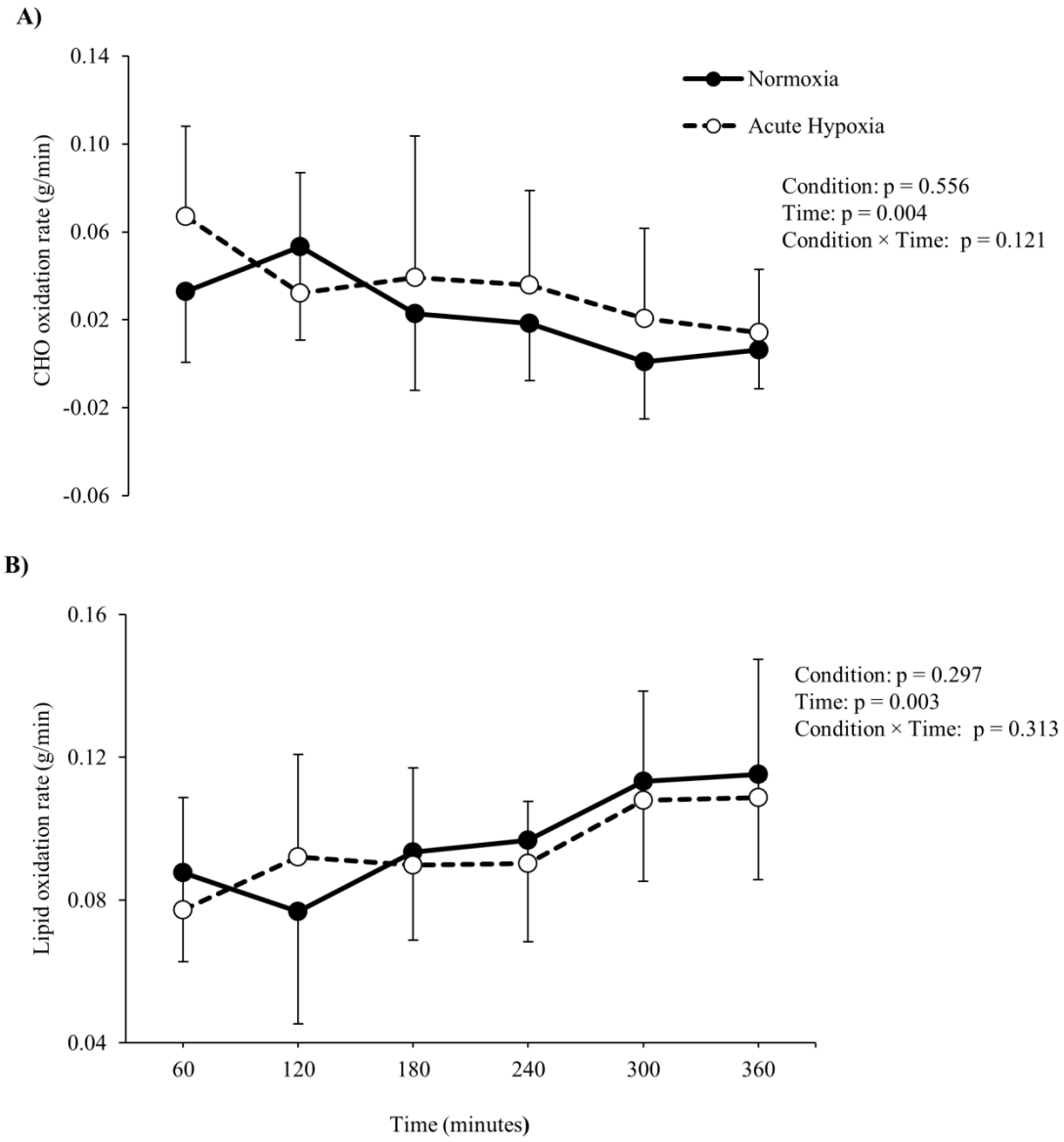

Figure 1. **(A)** Carbohydrate (CHO) oxidation rate, and **(B)** Lipid oxidation rate measured for 6h during normoxia and acute hypoxia sessions in young healthy men in fasting state. Values are mean ± standard deviation.

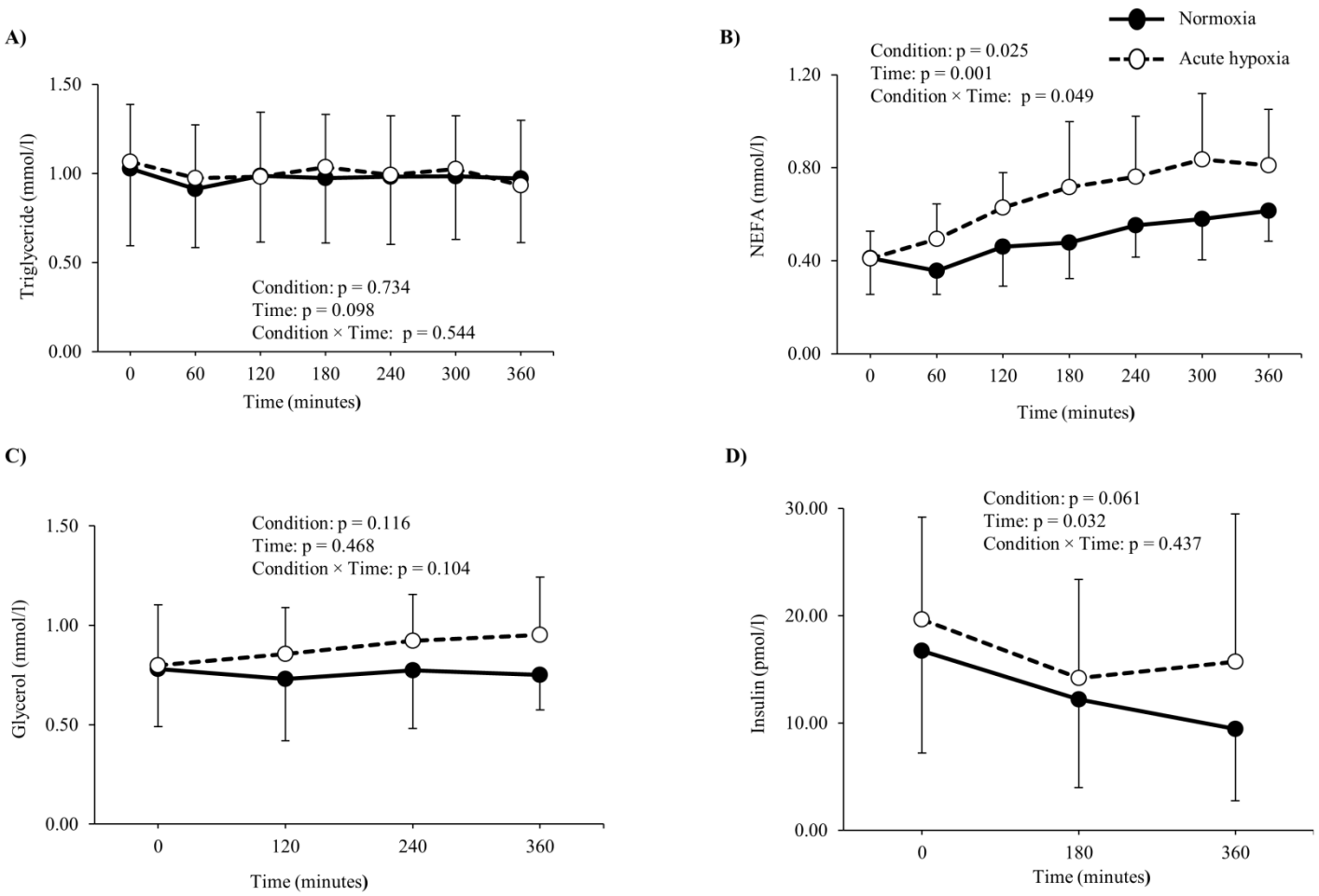

Figure 2. Effect of normoxia or acute hypoxia on fasting plasma **(A)** Triglyceride, **(B)** Non-esterified fatty acids (NEFA), **(C)** Glycerol, and **(D)** Insulin levels in healthy men. Values are mean ± standard deviation.

CHAPTER 4: THESIS DISCUSSION

4.1 Summary

Hypoxia is well recognized to induce many rescue pathways including augmenting glycolytic flux and reducing oxidative glucose oxidation, mainly catalyzed by changes orchestrated by the transcription factor hypoxia inducible factor-1 (HIF-1) (Semenza 1999, 2014, 2017). Less emphasis has been given on the impact of hypoxia on lipid mobilization and storage functions, key determinants in the development of metabolic disorders (DeFronzo 2004, Lelliott and Vidal-Puig 2004, Slawik and Vidal-Puig 2006). There is strong evidence from animal studies that hypoxia is noted in adipose tissue as the mass of the organ excessively expands and, in turn, exacerbates some adipose tissue functions (Hosogai et al. 2007, Rausch et al. 2008, Trayhurn and Alomar 2015). Whether hypoxia exposure, which could be derived from reduced environmental O_2 availability, disease or a combination of both, affects adipose tissue lipid storage and mobilization functions in humans are not well-known. Using *in vitro* and *in vivo* approaches, this thesis aimed at characterizing the effects of hypoxia on human adipose tissue lipid storage and lipid mobilization functions. These were:

1) How hypoxia affects LPL activity, the expression of genes involved in lipid storage and lipid mobilization, as well as lipolysis on differentiated human preadipocytes?

2) Does acute intermittent hypoxia affect plasma TG and adipose tissue LPL activity and lipolysis in healthy men in postprandial state?

3) Does an acute hypoxia exposure affect plasma TG levels in fasting healthy humans?

Table 1. Summary of the main thesis findings:

Study I	1. Both acute (t=24h, after differentiation) and chronic exposure (t=14d, during differentiation) to hypoxia (severe hypoxia 3% O_2, mild hypoxia 10% O_2, and control 21% O_2) has a concentration-dependent inhibiting effect on LPL activity. 2. Acute exposure to mild hypoxia stimulates the expression of lipid storage genes (FAS, DGAT2, and ChREBP) while chronic exposure to severe hypoxia inhibits gene expression of lipid storage (FAS, ACC, ChREBP, DGAT1, and DGAT2), and lipid mobilization (ATGL and HSL). 3. Acute hypoxia has a concentration-dependent stimulating effect on basal, but not isoproterenol-induced lipolysis while chronic hypoxia has an inhibiting effect on isoproterenol-induced, but not basal lipolysis.
Study II	Acute exposure to intermittent hypoxia (t=6h) was sufficient to increase postprandial NEFA levels, as well as insulin levels, but did not alter circulating TG or subcutaneous adipose tissue LPL activity and/or adipocyte lipolysis *ex vivo*.
Study III	Acute hypoxia (t=6h) progressively increases fasting NEFA and glycerol levels, suggesting increased adipose tissue lipolysis, but do not alter circulating TG concentrations nor post-heparin plasma lipolytic activity.

O_2: oxygen. TG: triglycerides. NEFA: non-esterified fatty acids. LPL: lipoprotein lipase.

To address these questions, three studies were performed, and the main findings are summarized in **Table 2**. The results of these studies have been expansively discussed in **Chapter 3**. However, the strengths, limitations, and future research were brief. Therefore, the following sections will consider the wide-ranging strengths, limitations, and future research in this area.

4.2 Strengths, limitations and future research

Study I: *Effects of different oxygen tensions on differentiated human preadipocytes lipid storage and mobilization functions*

The goal of this study was to investigate *in vitro* the lipogenic and lipolytic responses of human differentiated preadipocytes exposed acutely (24h) and chronically (14d) to control (21% O_2), mild (10% O_2), and severe hypoxia (3% O_2). Results indicate that 1) hypoxia dose-dependently inhibits LPL activity; 2) acute mild hypoxia seems to partly stimulate the *de novo* lipogenic pathway while severe or sustained hypoxia appears to repress DNL; 3) acute hypoxia has a concentration-dependent stimulating effect on basal but not isoproterenol-stimulated lipolysis; and 4) chronic hypoxia inhibits isoproterenol-stimulated but not basal lipolysis (**Chapter 3**). Therefore, both acute and chronic hypoxia (3%, and 10% O_2) appears to affect human adipose tissue lipid storage and mobilization functions, but in a different manner. Our observations suggest that hypoxia (3%, and 10% O_2) may impair adipose tissue lipid metabolism and expose other organs such as the heart, liver, and skeletal muscles to an excess of lipids and favor the risk of developing metabolic disorders, such as Type 2 diabetes and CVD.

Strengths

Our study was conducted *in vitro* using human subcutaneous preadipocytes primary cell culture. *In vitro* techniques have been validated as biologically relevant model to predict *in vivo* process (Cross and Bayliss 2000, BéruBé et al. 2010). The primary cells used in our study were asexual diploid cells obtained directly from two individuals. 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). The specific use of primary human preadipocytes in our studies has the advantage to reflect the human *in vivo* context better than murine (ex. 3T3-L1) cell lines, as animal cells are aneuploid while human cells are diploid. In addition, using an *in vitro* technique allowed us to control the growth conditions, thus eliminating many confounding variables that exist *in vivo*, for instance genetic backgrounds between individuals, among others (Cross and Bayliss 2000, BéruBé et al. 2010, Myre 2014).

We used three different O_2 concentrations (21%, 10%, and 3%) in the study based on following arguments. First, cell culture has traditionally been done under 21% O_2, so this O_2 concentration has been used for the control condition (Famulla et al. 2012, O'Rourke et al. 2013, Trayhurn 2013). We used 10% O_2 as a mildly hypoxic condition since it has been argued that the real O_2 tension in human adipose tissue is closer to 10% O_2 (Goossens et al. 2011, Trayhurn 2013). Alternatively, 10% O_2 could also be considered a more physiologically relevant control condition. Finally, we chose 3% O_2 as the severe hypoxic condition based on the fact that other studies have used O_2 concentrations ranging from 1-5% O_2 to study the effects of hypoxia *in vitro* (Famulla et al. 2012, O'Rourke et al. 2013, Trayhurn 2013).

Limitations and future research

This two-dimensional cell culture does not encompass the three dimensional complexity of multi-cellular organisms. 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 endothelial cells, and macrophages (Grimm 2004, BéruBé et al. 2010, Myre 2014). It would be interesting to determine their response to the similar type of experimental conditions.

Both acute and chronic exposure to hypoxia had a concentration-dependent inhibiting effect on LPL activity. The reduction on adipocyte LPL activity may be explained by the upregulation of an important post-translational repressor of LPL, ANGPTL-4, during hypoxia exposure (Drager et al. 2012). This hypothesis remains to be tested in humans. One possibility to isolate the specific role of ANGPTL-4 expression, could be by using silencing RNA (siRNA), a recently developed tool for gene silencing (Makoveichuk et al. 2013). This technique consists of introducing a short RNA molecule into the cells that selectively targets and destroys specific mRNA transcripts. We have attempted performing this technique with primary human preadipocytes, but without success. As compared to the other cells line in which the silencing RNA has been validated (ex. 3T3-L1), differentiated human preadipocytes, as used in our experiments, have substantial lipid droplets which exacerbates the RNA silencing process. Further attempts at an earlier stage of differentiation of human preadipocytes, where lipid droplets are less prominent, should be tested.

It appears clearly that chronic exposure to severe hypoxia (3% O_2) induced a decrease in the expression of several lipogenic genes, namely FAS, ACC, ChREBP, DGAT1 and DGAT2 while there was only minor effect after chronic exposure to mild hypoxia (10%O_2) (decrease in DGAT1 mRNA expression), acute exposure to severe hypoxia (increase in ChREBP mRNA expression), and acute exposure to mild hypoxia (increase in FAS, DGAT2 and ChREBP mRNA expression). These results indicate that any greater hypoxic challenge, in terms of severity or duration, has no effect or even decrease the lipogenic potential of human adipocytes. O'Rourke et al. (O'Rourke et al. 2013) similarly observed that a 3 day exposure to severe hypoxia (1% O_2) inhibits FAS mRNA expression by 20-30% in human visceral adipocytes and attributed part of the reduction in lipogenesis to a decrease in glutamine metabolism and hexosamine production.

However, the physiological mechanisms responsible for the reduced lipogenic potential in response to severe or sustained hypoxia are largely unknown and warrant further studies. It could be hypothesized that lipogenesis, being an anabolic process, requires ATP and therefore O_2. It is not unlikely that the shutdown of the ATP-consuming lipogenesis pathway in response to low O_2 condition occurs to preserve energy for cell survival (Liu et al. 2006). This is supported by observations by Liu et al. (Liu et al. 2006) who showed that hypoxia activates adenosine monophosphate-activated protein kinase (AMPK), a well-known regulator of lipogenesis. It would have been interesting to quantify whether AMPK was activated, using western blot, in our experimental conditions.

As for lipogenesis, the effects of chronic hypoxia on lipolysis somewhat diverge from the effects of acute hypoxia. Chronic hypoxia reduced the lipolytic response to isoproterenol, without affecting basal lipolysis. It has been suggested that acute induction of lipolysis by β-adrenergic stimulation increases O_2 consumption (Yehuda-Shnaidman et al. 2010). Since lipolytic rates were assessed under hypoxia, it is possible that the lack of O_2 *per se* may have blunted the ability of adipocytes to increase their lipolytic rate. Interestingly, adipocytes from individuals with severe obesity also respond poorly to catecholamine stimulation, which could possibly be explained by a decrease in β2-adrenergic receptor density (Reynisdottir et al. 1994, Large et al. 2004). Further studies will need to be conducted, using radioligand as iodo cyanopindolol to better estimate β-adrenoceptor binding capacity and elucidate how chronic hypoxia can reduce isoproterenol-induced lipolysis without affecting basal lipolysis and to examine if the same mechanisms can explain the catecholamine resistance observed in adipocytes from individuals with obesity.

Study II: *Effects of acute intermittent hypoxia on human adipose tissue lipid storage and mobilization functions.*

To explore whether the inhibitory effect of hypoxia on adipose tissue functions are noticeable in young healthy men exposed for 6 hours to acute intermittent hypoxia, an experimental model that has been proposed to study the metabolic effects of OSA. Acute exposure to intermittent hypoxia was sufficient to alter postprandial NEFA levels, as well as glucose and insulin levels, but did not alter circulating TG nor subcutaneous adipose tissue LPL activity and/or adipocyte lipolysis *ex vivo* (**Chapter 3**). Hypoxia increased plasma NEFA concentrations by 33% after 360 minutes compared to baseline. Despite no effect on adipose tissue lipolysis *ex vivo*, the elevated plasma NEFA levels observed during acute intermittent hypoxia could still come from an increase in sympathetic activation (Hansen and Sander 2003, Prabhakar and Kumar 2010), which should have been less present in the normoxia session. Sympathetic activation is well recognized to impair insulin sensitivity (Lambert et al. 2015). In this regard, Peltonen et al. (Peltonen et al. 2012) have elegantly demonstrated that the SNS activation derived from an acute reduction in O_2 availability disrupts insulin sensitivity in humans. Consistent with this observation, insulin levels in our study were 80% greater at 30 minutes compared to the baseline during hypoxia exposure.

Strengths

Intermittent hypoxia models are one of the most commonly used research models of OSA (Young et al. 2002, Government of Canada 2010). It has been employed in rodents (Drager et al. 2012) and humans (Louis and Punjabi 2009). In humans, subjects have to wear a well-fitted oro-nasal mask with a two-way Hans Rudolph non-rebreathing valve connected to an inspiratory line, as reported by Louis et al. (Louis and Punjabi 2009). During intermittent hypoxia sessions,

pressurized medical nitrogen (N_2) is intermittently introduced in the inspiratory line. During normoxia session, ambient air only is provided. Intermittent hypoxia models vary in both frequency and severity of the hypoxia stimulus. In our experiment, oxyhemoglobin saturation (SpO_2) was allowed to drop to 85%, at which point the flow of N_2 was stopped until the SpO_2 returned to the pre-exposure values (~98%). This experimental setup allowed us to produce 17.3 ± 3.8 hypoxia events per hour, which is comparable to moderate OSA (Young et al. 2002, Government of Canada 2010). Therefore, this approach has been exclusively employed to study metabolic outcomes of intermittent hypoxia and OSA (Louis and Punjabi 2009, Drager et al. 2010). Overall, this study provided a better understanding of the effects of OSA on lipid storage and mobilization functions, and helped us refine the potential link between hypoxia and metabolic disease risks for individuals living with sleep apnea and/or chronic obstructive.

Limitations and future research

We observed that acute exposure to intermittent hypoxia (t=6h), a simulation model of OSA, was not sufficient to alter postprandial circulating TG in healthy men. These results are not consistent with previous animal studies that demonstrated that chronic exposure to intermittent hypoxia induces substantial rise in circulating TG by increasing hepatic TG secretion in the fasted state and delaying TG clearance in the postprandial state (Drager et al. 2012, Yao et al. 2013). With regards to our *in vivo* study, the duration of the hypoxic exposure was limited to 6 hours to limit the burden, and potential side-effects on the hypoxia naïve participants. The severity of the intermittent hypoxia was equivalent to moderate OSA (Young et al. 2002, Government of Canada 2010). Whether a prolonged exposure to intermittent hypoxia could induce significant changes in TG metabolism remains to be tested in humans.

A second limitation regards the homogeneity of our study sample, which consisted exclusively of healthy young men. This prevents the generalization of our observations to metabolically deteriorated individuals, such as individuals with OSA. Individuals with OSA are likely exposed to intermittent hypoxia on a daily basis, and a large proportion of them exhibit metabolic complications (Drager et al. 2010) – increased adiposity, dyslipidemia, and insulin resistance (consequently of OSA or not) – that may synergistically exacerbate the negative lipid-altering effects of intermittent hypoxia. Part of the basis for this proposition lies on the limited vascularization of adipose tissue as well as the absence of increase in blood flow in the tissue (Newman et al. 2005, Drager et al. 2010, Trayhurn and Alomar 2015). Whether individuals characterized by greater body fat % or by some lipid metabolism impairment could be affected differently by intermittent hypoxia remains to be addressed.

Finally, we observed that acute exposure to intermittent hypoxia, a simulation model of OSA, was not sufficient to alter postprandial subcutaneous adipose tissue lipid storage (LPL) and/or adipocyte lipolysis *ex vivo* in healthy men. The periumbilical region was chosen as the sampling site because it is well-known that 45-50% of the TG derived from any lipid we ingest are absorbed by subcutaneous abdominal adipose tissues (McQuaid et al. 2011, Koutsari et al. 2011). This makes subcutaneous abdominal adipose tissue the most important tissue responsible for the storage of postprandial TG and mobilization of NEFA in humans. The remaining of the postprandial TG clearance and mobilization of NEFA will be assured by the action of the LPL activity and lipolysis derived from various other sites such as the subcutaneous femoral and visceral adipose tissues as well as the heart. Future studies remain to be performed to investigate how these other sources of LPL activity and lipolysis could be affected by intermittent hypoxia.

Study III: *Effects of acute normobaric hypoxia on plasma triglyceride levels in fasting healthy men.*

This study aimed at determining the effect of an acute 6-hour bout of hypoxia on plasma TG concentrations in fasting healthy young males. Plasma TG are an important risk factor in the development of chronic diseases such as type 2 diabetes and cardiovascular diseases (Kalofoutis et al. 2007, Miller et al. 2011). We show that acute hypoxia progressively increases fasting NEFA (95% greater increase) and glycerol (33% increase) levels, suggesting an increased in adipose tissue lipolysis, but do not alter post-heparin plasma lipolytic activity nor circulating TG concentrations in young men with normal adiposity level (**Chapter 3**). Despite no changes in plasma TG concentrations, the increase in plasma NEFA concentrations from baseline to 360 minutes was 95% greater during hypoxia compared to normoxia, suggesting an increased in adipose tissue lipolysis. It is worth noting that NEFA concentrations showed no evidence of stabilization after 6 hours, which suggest that higher plasma NEFA concentrations could have been reached given a longer exposure. Exposure to reduced partial pressure of O_2 is well recognized to increase sympathetic activation (Hansen and Sander 2003, Prabhakar and Kumar 2010), which is an important activator of adipose tissue lipolysis. Consistently, the 24% increase in heart rate and the 33% increase in glycerolemia after 360 minutes of hypoxia exposure are strong indicators that our experimental hypoxia exposure induced sympathetic arousal and stimulated lipolysis. Sympathetic activation is also well recognized to impair insulin sensitivity (Lambert et al. 2015). Consistent with this observation, insulin levels in this study were 66% greater after 6 hours of hypoxia exposure despite similar glucose levels. This apparent reduction in insulin sensitivity, if present at the adipose tissue level, also may have contributed to a hypoxia-induced increase in lipolysis by lifting the inhibitory effect of insulin.

Strengths

Exposure to hypoxia in an environmental chamber room is one of the most commonly used research model of exposure to high altitude condition (Gallagher et al. 2014, Ofner et al. 2014). It has been predominantly employed in humans (Gallagher et al. 2014, Ofner et al. 2014). Environmental chamber rooms vary in the severity of the hypoxia stimulus. In our experiment, during hypoxia, O_2 extractors (CAT12, Altitude Control Technologies, Lafayette, Colorado, USA) were connected to the environmental chamber, which allowed for a stabilized FIO_2 level at 0.12, equivalent to 4200 m altitude. During the normoxia sessions, only ambient air was used ($FIO_2 = 0.21$). The main advantage of exposure to hypoxia in an environmental chamber room is to control for confounding factors such as physical activity, environmental conditions and/or diet that may explain the inconsistency in the literature related to the impact of altitude exposure on TG in humans.

Limitations and future research

First, the main endpoint, plasma TG concentrations, does not provide all the information regarding TG metabolism. The use of stably-labelled tracer, such as labelled glycerol or leucine, and mass-spectrometry, could allow to better estimate TG production and clearance rates and provide a more detailed picture of the effect of hypoxia on TG metabolism (Adiels et al. 2015).

We hypothesized that combined effects of fasting (low insulinemia) and hypoxia (sympathetic arousal) would increase circulating NEFA concentrations to the liver, which could in turn induce increase in TG levels. Our results indicated that fasting TG levels do not change in response to a 6-hour exposure to normobaric hypoxia. Results from previous studies have reported increases (+ 44% on average after 9 days at 4265 m (Whitten and Janoski 1969), + 81% on average after

40 days at 8848 m (Young et al. 1989), + 47% on average after 8 months at 3550 m (Siqués et al. 2007)), in fasting plasma TG after only prolonged exposure to hypoxia in humans. With regards to our *in vivo* study, the duration of the hypoxic exposure was limited to 6 hours to limit the burden, fasting time and potential side-effects on the hypoxia naïve participants. Whether a prolonged exposure could induce significant changes in plasma TG concentrations remains to be tested.

Another possible explanation for the absence of shift in plasma TG despite an increase in NEFA availability is that NEFA are not utilized directly as an energy substrate in organs or for VLDL assembly in the liver, but first enter a temporary and probably expendable intracellular TG pool (Gibbons et al. 2004). This buffering capacity of the liver and/or peripheral organs could delay an increase in hepatic TG output in response to a rise in plasma NEFA and mitigate the effects of acute hypoxia on TG metabolism in humans. It would be interesting to determine intracellular TG, using ^{1}H magnetic resonance spectroscopy (Szczepaniak et al. 1999), to the same experimental conditions. The limiting factor is the high-cost of the equipment.

The peripheral clearance of VLDL-TG, is catalyzed mainly by the LPL and the HL, the activity of both being assessable in post-heparin plasma (Després et al. 1999). We found no difference in post-heparin plasma lipase activity after 6-hour of hypoxia exposure, which suggests that hypoxia does not acutely effect TG clearance in healthy young individuals. In our study, plasmatic lipolytic activity was measured using the Enzchek fluorescent TG-analog substrate on blood samples (Basu et al. 2011). This substrate is recognized by both LPL and HL activity, so we were not able to distinguish between LPL and HL activity. Whether changes in LPL activity are, in these conditions, counterbalanced by changes in the activity of other lipases, such as HL, will need to be clarified. Addition of sodium chloride (NaCl) usually inhibits LPL activity and

able to determine HL activity. However, in our experiment, there was no inhibition of LPL activity by NaCl, so we were not able to distinguish between LPL and HL activity (Yagyu et al. 2003). Further studies using triolein substrate, and addition of NaCl is warranted to test this hypothesis (Krauss et al. 1974, Huttunen et al. 1975, Henderson et al. 1993, Yagyu et al. 2003).

4.3 Thesis Conclusions

The findings presented in this thesis demonstrate the following: First, the *in vitro* studies on differentiated human preadipocytes suggest that hypoxia dose-dependently inhibit LPL activity but that acute mild hypoxia seems to partly stimulate the *de novo* lipogenic pathway while severe or sustained hypoxia appear to repress DNL. Additionally, acute hypoxia had a concentration-dependent stimulating effect on basal but not isoproterenol-stimulated lipolysis while chronic hypoxia reduced the lipolytic response to isoproterenol, without affecting basal lipolysis. Second, *in vivo* observations show that an acute session of intermittent hypoxia significantly increases postprandial NEFA levels, but not postprandial circulating TG, adipose tissue LPL activity, or adipocyte lipolysis *ex vivo*, in healthy young men. Finally, acute exposure to normobaric hypoxia increases adipose tissue lipolysis but the resulting increase in fatty acid availability does not translate into elevated circulating TG concentrations in fasting healthy men. In conclusion, our observations suggest that an exposure to reduced O_2 levels impairs human adipose tissue storage and/or mobilization functions, a phenomenon known in the development of metabolic disorders, such as Type 2 diabetes and CVD.

CHAPTER 5: REFERENCES

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APPENDIX

Appendix A: Notices of ethical approval for thesis studies

Numéro de dossier: H05-13-13B

Date (mm/jj/aaaa): 04/12/2017

Université d'Ottawa
Bureau d'éthique et d'intégrité de la recherche

University of Ottawa
Office of Research Ethics and Integrity

Certificat d'approbation déontologique

CÉR Sciences et science de la santé

Chercheur principal / Superviseur / Co-chercheur(s) / Étudiant(s)

Prénom	Nom de famille	Affiliation	Rôle
Pascal	Imbeault	Sciences de la santé / Activité physique	Chercheur principal
Bimit	Mahat	Sciences de la santé / Activité physique	Co-chercheur
Jean-François	Mauger	Sciences de la santé / Activité physique	Co-chercheur
Etienne	Chassé	Sciences de la santé / Activité physique	Assistant de recherche

Numéro du dossier: H05-13-13B

Type du projet: Professeur

Titre: The effects of acute hypoxia on postprandial metabolism

Date de renouvellement (mm/jj/aaaa)	Date d'expiration (mm/jj/aaaa)	Approbation
04/02/2017	04/01/2018	Renouvellement

Conditions Spéciales / Commentaires:
N/A

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La présente confirme que le Comité d'éthique de la recherche (CER) de l'Université d'Ottawa identifié ci-dessus, opérant conformément à l'Énoncé de politique des Trois conseils et toutes autres lois et tous règlements applicables de l'Ontario, a examiné et approuvé la demande d'approbation éthique du projet de recherche ci-nommé. L'approbation est valide pour la durée indiquée plus haut et est sujette aux conditions énumérées dans la section intitulée "Conditions Spéciales / Commentaires".

Lors de l'étude, le protocole ne peut être modifié sans approbation préalable écrite du CER sauf si le participant doit être retiré en raison d'un danger immédiat ou s'il s'agit d'un changement ayant trait à des éléments administratifs ou logistiques de l'étude comme par exemple un changement de numéro de téléphone. Les chercheurs doivent aviser le CER dans les plus brefs délais de tout changement pouvant augmenter le niveau de risque aux participants ou affecter considérablement le déroulement du projet. Ils devront aussi rapporter tout évènement imprévu et / ou dommageable et devront soumettre toutes les nouvelles informations pouvant nuire à la conduite du projet et/ou à la sécurité des participants. Toutes modifications apportées au projet, aux lettres d'information / formulaires de consentement ainsi qu'aux documents de recrutement doivent être soumises pour approbation en utilisant le document intitulé "Modification au projet de recherche" au: https://recherche.uottawa.ca/deontologie/formulaires.

Veuillez soumettre un rapport annuel au Bureau d'éthique quatre semaines avant la date d'échéance indiquée afin de renouveler de l'approbation éthique. Afin de fermer le dossier, un rapport final doit être soumis. Ces documents sont disponibles en ligne au: https://recherche.uottawa.ca/deontologie/formulaires.

Pour toutes questions, vous pouvez communiquer avec le Bureau d'éthique en composant le poste 5387 ou par écrit à: ethique@uOttawa.ca.

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Appendix B: Final published version of thesis article #2

Mahat et al. J Transl Med (2016) 14:212
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Journal of Translational Medicine

RESEARCH Open Access

Effects of acute hypoxia on human adipose tissue lipoprotein lipase activity and lipolysis

Bimit Mahat, Étienne Chassé, Jean-François Mauger and Pascal Imbeault*

Abstract

Background: Adipose tissue regulates postprandial lipid metabolism by storing dietary fat through lipoprotein lipase-mediated hydrolysis of exogenous triglycerides, and by inhibiting delivery of endogenous non-esterified fatty acid to nonadipose tissues. Animal studies show that acute hypoxia, a model of obstructive sleep apnea, reduces adipose tissue lipoprotein lipase activity and increases non-esterified fatty acid release, adversely affecting postprandial lipemia. These observations remain to be tested in humans.

Methods: We used differentiated human preadipocytes exposed to acute hypoxia as well as adipose tissue biopsies obtained from 10 healthy men exposed for 6 h to either normoxia or intermittent hypoxia following an isocaloric high-fat meal.

Results: In differentiated preadipocytes, acute hypoxia induced a 6-fold reduction in lipoprotein lipase activity. In humans, the rise in postprandial triglyceride levels did not differ between normoxia and intermittent hypoxia. Non-esterified fatty acid levels were higher during intermittent hypoxia session. Intermittent hypoxia did not affect subcutaneous abdominal adipose tissue lipoprotein lipase activity. No differences were observed in lipolytic responses of isolated subcutaneous abdominal adipocytes between normoxia and intermittent hypoxia sessions.

Conclusions: Acute hypoxia strongly inhibits lipoprotein lipase activity in differentiated human preadipocytes. Acute intermittent hypoxia increases circulating plasma non-esterified fatty acid in young healthy men, but does not seem to affect postprandial triglyceride levels, nor subcutaneous abdominal adipose tissue lipoprotein lipase activity and adipocyte lipolysis.

Keywords: Intermittent hypoxia, Obstructive sleep apnea, Adipose tissue metabolism, Postprandial lipemia, Cardiovascular disease

Background

Obstructive sleep apnea (OSA) is a prevalent sleep disorder affecting approximately 5–15 % of middle-aged and older adults in the general population [1]. Individuals with OSA experience short periods of hypopnea, inducing intermittent hypoxia-hypercapnia/normoxia cycles. The most salient symptom of OSA is excessive daytime sleepiness, but its most important health consequence is an approximate two-fold increased risk of developing cardiovascular disease (CVD) such as coronary artery disease, heart failure, or stroke [2]. The link between OSA and CVD could be explained by the fact that OSA may disturb lipid metabolism and lead to a deteriorated blood lipid profile. It has been shown that individuals with OSA display increased triglyceridemia (by ~30 %), independent of age and body mass index, compared to individuals without OSA [3].

Adipose tissue plays a central role in energy substrate homeostasis by acting as a crucial regulator of whole-body lipid flux. More specifically, in response to metabolic demand, triglyceride (TG) stored within adipocytes can be hydrolyzed into fatty acids and glycerol to be

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released for use by non-adipose organs. Postprandially, the transport of lipoprotein lipase (LPL) from intracellular vacuoles to the capillaries endothelium promotes the hydrolysis of dietary TG and subsequent uptake of dietary fatty acids within adipocytes [4, 5]. The proper regulation of lipid uptake and secretion by the adipose tissue is thought to be critical to limit ectopic fat storage in metabolically important tissues, namely the liver, skeletal muscles, and pancreatic beta cells, and to prevent chronic disorders such as type 2 diabetes and CVD [6, 7].

Recent animal studies demonstrated that chronic intermittent [8, 9] and acute hypoxia [10] increase hepatic TG secretion in the fasted state and delay TG clearance in the postprandial state. These changes appear to be caused, in part, by (a) an increase in lipid influx to the liver due to an increase in adipose tissue lipolysis and by (b) a suppression of LPL activity by more than 50 %. While the increase in adipose tissue lipolysis has been linked to the increase in sympathetic drive observed during hypoxia, the reduction in adipose tissue LPL activity appears to be explained by the upregulation of an important post-translational repressor of LPL, angiopoietin-like protein 4 (ANGPTL4) [9].

Despite evidence from animal studies indicating that hypoxia considerably affects adipose tissue functions, blood lipid profile, and potentially the risk of CVD or type 2 diabetes in OSA patients, data regarding these effects in humans is crucially lacking. Therefore, the objective of this study was to investigate the effects of hypoxia on human adipose tissue LPL activity and adipocyte lipolysis. We hypothesize that: (1) In differentiated human preadipocytes, acute exposure to hypoxia inhibits LPL activity, and (2) In humans, acute intermittent hypoxia leads to an exaggerated elevation in postprandial TG concentrations consequent to an increase in adipocyte lipolysis and/or an impairment in subcutaneous abdominal adipose tissue LPL activity.

Methods

In vitro experiments

Culture of human preadipocytes

Cryopreserved subcutaneous abdominal preadipocytes from two Caucasian female (average age: 39 y; mean body mass index: 22.74 kg/m^2) were obtained from Zenbio (NC, USA) and differentiated according to manufacturer's instructions [11]. Briefly, preadipocytes were plated at a density of 4×10^4 cells/cm^2 in 24-well plates, and proliferated in preadipocytes medium (PM-1) for 48 h, or until confluence was reached. Differentiation was induced by substituting the culture media for adipocyte differentiation medium (DM-2) in which cells were maintained for 7 days. Cells were then fed by replacing the culture medium with the adipocyte maintenance medium (AM-1), and maturation was continued for another week. Fourteen days post-induction, cells were transferred to basal medium (BM-1) and incubated in either hypoxic (3 % oxygen) or normoxic (21 % oxygen) conditions [12], for 24 h. No cell lost was observed at the end of each treatment. After treatments, media were collected and cells were washed three times with phosphate buffer saline (PBS). To assess LPL activity, cells were incubated for 30 min in their respective oxygen conditions, in presence of BM-1 containing 100 U/ml heparin. BM-1/heparin media were collected, cells were wash three times with PBS and lysed with RLT buffer (QIAGEN) containing 10 % β-mercaptoethanol.

RNA Isolation and RT-PCR

Total RNA was extracted from cell lysates using QIAGEN RNeasy Mini kits, following the manufacturer's instructions. Complementary DNA was prepared from 300 ng of total RNA using QIAGEN reverse transcriptase kit, following elimination of genomic DNA using QIAGEN gDNA WipeOut. Since there is no discrepancy between protein level and mRNA expression of Angiopoietin-like 4 (ANGPTL4), only the gene expression was determined [9]. Gene expression was determined by real-time PCR using Eva Green Master Mix (Montreal Biotech) on a Rotor-Gene. Quantitect primers (forward and reverse) for ANGPTL4, metallothionein-3 (MT3), and β-actin were purchased from QIAGEN, with β-actin serving as the reference gene. Delta-delta CT (cycle threshold) analyses were conducted using the Rotor-Gene 6000 software version 1.7.

LPL activity

LPL activity in differentiated preadipocytes was measured in 50 µl of BM-1-Heparin using the EnzChek Lipase Substrate (Thermo Fisher Scientific), a fluorescent triacylglycerol analog, at a final concentration of 0.62 µM in presence of 18-carbon zwittergent (0.0125 %), 0.15 M NaCl and 20 mM Tris–HCl pH 8. Fluorescence emission kinetics were followed over 1 h at 37 °C and fluorescence from blank wells was subtracted. Average blank-adjusted RFU (relative fluorescence units) are reported here. All samples from an identical experiment were assessed simultaneously, alongside positive controls containing bovine LPL. LPL activity in adipose tissue biopsies was determined similarly, excepted that LPL was first extracted from thawed subcutaneous abdominal adipose tissue samples by incubation at 28 °C for 40 min in Krebs–Ringer buffer containing 1 % BSA (bovine serum albumin) and 0.05 mg/ml heparin as previously described [13, 14].

In vivo experiments

Subjects

Ten healthy young men were recruited from the University of Ottawa population. Study subjects provided written consent and the study protocol was approved by the Research and Ethics Board of the University of Ottawa. Exclusion criteria included: history of physician-diagnosed asthma or other respiratory illness, hypertension, CVD, diabetes, habitual sleep duration of less than 7 h per night, habitual bed time occurring after midnight, shift work, and current smoking habit.

Anthropometric measurements

Body weight was determined with a standard beam scale (HR-100, BWB-800AS; Tanita, Arlington Heights, IL) and height was measured using a standard stadiometer (Perspective Enterprises, Portage, Michigan, USA). Waist circumference was measured following World Health Organization procedure. Percentage of fat mass (%FM), total fat mass (FM) and fat free mass (FFM) were measured using dual energy X-ray absorptiometry (DXA) (General Electric Lunar Prodigy, Madison, Wisconsin; software version 6.10.019). Resting energy expenditure (REE) was measured by indirect calorimetry using a Vmax Encore 29 System metabolic cart (VIASYS Healthcare Inc, Yorba Linda, CA).

Experimental protocol

This was a randomized crossover study consisting of two experimental sessions. Prior to each experimental session, volunteers were counseled to sleep at least 7 h per night, to restrain from any exercises and caffeine for at least 24 h, and to consume a provided standardized evening dinner between 7:00 and 8:00 PM (lasagna of 3220 kJ or 770 kcal; 42 % from carbohydrates, 28 % from fat, and 30 % from protein). On study days, volunteers presented themselves at the laboratory at 7:30 AM after a 12-h overnight fast. Weight measurements were performed before an intravenous line was inserted in the antecubital vein for blood sampling and kept patent with a continuous infusion of 0.9 % saline. A baseline subcutaneous abdominal adipose tissue biopsy (detailed below) was then performed. Volunteers were thereafter asked to consume a fat-rich liquid meal (59 % of calories from fat, 28 % from carbohydrates and 13 % from protein) providing one-third of their estimated daily energy expenditure (obtained by indirect calorimetry during a preliminary session) times a physical activity factor of 1.375 [15], and were then exposed to either intermittent hypoxia or to ambient air (normoxia) for 6 h. Volunteers remained in a semirecumbent position, and occupied themselves by watching television. Sleep was not allowed. Oxyhemoglobin saturation and heart rate were continuously monitored by pulsed oximetry. A second adipose tissue biopsy was performed 3 h after meal ingestion.

OSA simulation (intermittent hypoxia)

Subjects had to wear a well-fitted oro-nasal mask with a two-way Hans Rudolph non-rebreathing valve connected to an inspiratory line, as reported by Louis et al. [16]. During normoxia session, ambient air only was provided. During intermittent hypoxia sessions, pressurized medical N_2 was intermittently introduced in the inspiratory line. Oxyhemoglobin saturation (SpO_2) was allowed to drop to 85 %, at which point the flow of N_2 was stopped until the oxyhemoglobin saturation returned to the pre-exposure values (~98 %). Intermittent hypoxia was well-tolerated and presented no adverse effects. This experimental setup allowed us to produce 17.3 ± 3.8 hypoxic events per hour, which is comparable to moderate OSA.

Fasting and postprandial plasma metabolic parameters

Plasma was obtained by centrifugation at 3000 rpm for 10 min at 4 °C immediately after blood collection. Commercially available colorimetric enzymatic assays were used to measure plasma total triglyceride, glucose, non-esterified fatty acid (NEFA) (Wako Chemicals USA Inc, VA, USA) and lactate concentrations (Eton Bioscience Inc. NL, USA). Commercially available enzyme-linked immunosorbent assay kits were used to determine insulin (EMD Millipore, MA, USA) and catecholamines (Rocky Mountain Diagnostics Inc, CO, USA), as previously described [17].

Subcutaneous abdominal adipose tissue biopsy

On both experimental sessions, two subcutaneous abdominal fat biopsies were performed, one before and one 3 h after meal ingestion. Biopsies were performed in the periumbilical region (within 4–6 cm), as previously described [13]. On the second experimental session, biopsies were performed 4 cm underneath the incisions made on the first session.

Adipocyte lipolysis

Immediately after the biopsy, roughly 100 mg of fresh adipose tissue, free of capillaries, were digested with collagenase (1 mg/ml) in 4 % BSA Krebs–Ringer buffer at 37 °C and filtered through a nylon mesh. Adipocytes were isolated by centrifugation (500 rpm for 2 min), and washed twice with BSA-Krebs–Ringer buffer. Adipocyte density was then adjusted to 500 adipocytes/50 µl. With constant stirring, 50 µl aliquots of adipocytes suspension were distributed in 1.5 ml Eppendorf tubes and incubated at 37 °C for 2 h in BSA-Krebs–Ringer buffer under 95 % O_2 in presence

of isoproterenol (0.001, 0.01, 0.1, 1 and 10 µM), epinephrine (0.001, 0.01, 0.1, 1 and 10 µM) and UK 14304 (0.0001, 0.001, 0.01, 0.1 and 1 µM). Epinephrine and UK 14304 tubes also contained adenosine deaminase (ADA). Lipolytic rate was determined by glycerol quantification using bioluminescence, as described by Mauriege et al. [18]. Adipocyte density (cells/50 µl) was determined by counting and averaging the number of adipocytes in five 50 µl samples collected throughout the distribution step. Results are presented as µmol of glycerol released by 1×10^6 adipocytes over 2 h. Adipocyte size was obtained by analysing 10× digital images of adipocytes loaded on a hemocytometer using the Infinity Capture and Analyse software (Lumenera Corporation, ON, Canada). Each average adipocyte diameter was computed from at least 150 random individual measurements.

Statistical analysis

SPSS version 12 for windows was used for data analysis (SPSS Inc. Chicago, IL, USA). Repeated measures analyses of variance (ANOVA) were performed with condition and time as within subject's parameters. Alpha was set at 0.05.

Results

LPL Activity in differentiated human preadipocytes

In vitro, hypoxia induced a significant 6-fold reduction ($p < 0.001$) in LPL activity (Fig. 1a). mRNA levels of ANGPTL4, a repressor of LPL activity, and MT3, a gene known to be highly induced by hypoxia, were increased by 27-fold ($p < 0.001$) and 70-fold ($p < 0.001$) respectively following hypoxia (Fig. 1b, c).

Subject characteristics

Metabolic and anthropometric characteristics of the 10 healthy men are represented in Table 1. Participants reported a good quality of sleep, according to the Pittsburgh Sleep Index (3.83 ± 2.71) [19]. On average, participants reported 7.3 h of sleep during the night prior to the experimental sessions. The average time between each experimental session was 7.4 days, and participants' weight (± 0.35 kg) did not differ between experimental sessions.

Oxyhemoglobin saturation and heart rate responses to intermittent hypoxia

Table 2 displays the variations in heart rate and oxyhemoglobin saturation during normoxia and intermittent hypoxia sessions. During intermittent hypoxia, an average of 17.3 ± 3.8 hypoxic cycles was induced per hour. Heart rate was significantly increased during hypoxic exposure, reaching an average peak increase of ~20 bpm.

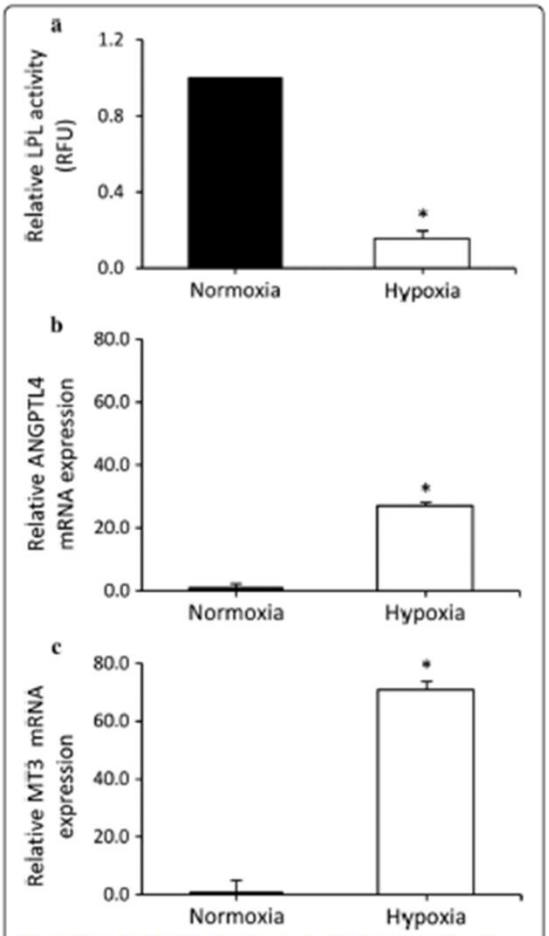

Fig. 1 Effect of normoxia (21 % oxygen) or hypoxia (3 % oxygen) on **a** lipopoprotein lipase activity, **b** Angiopoietin like 4 (ANGPTL4) gene expression and **c** metallothionein-3 (MT3) gene expression in differentiated human preadipocytes. Results are from 3 independent experiments performed in triplicate. Values are mean ± standard deviation. Significant difference between experimental sessions at $^{*}p < 0.001$

Plasma metabolic parameters

Postprandial plasma TG, glucose, lactate, insulin, and NEFA levels during normoxia and intermittent hypoxia sessions are depicted in Fig. 2. Postprandially, TG levels increased significantly (time effect, $p < 0.001$) but did not differ between normoxia and intermittent hypoxia sessions (Fig. 2a). Regardless of time, glucose and lactate were significantly greater during intermittent hypoxia than normoxia (condition effect, $p < 0.05$). Both variables evolved in a similar manner over time (time effect, $p < 0.01$) (Fig. 2b, c).

Table 1 Characteristics of the participants (n = 10 men)

Variable	Mean ± standard deviation
Age (y)	22.8 ± 2.8
Body weight (kg)	84.5 ± 9.8
Height (cm)	181.7 ± 4.7
Body mass index (kg/m^2)	25.6 ± 2.3
Waist circumference (cm)	84.9 ± 5.1
Fat mass (kg)	12.5 ± 4.5
Lean mass (kg)	69.4 ± 11.2
Body fat (%)	15.3 ± 4.1
Subcutaneous abdominal adipocyte diameter (μm)	72.8 ± 5.7

Table 2 Summary of heart rate and oxyhemoglobin saturation (SpO_2) during normoxia and intermittent hypoxia sessions

		Normoxia	Intermittent hypoxia
	Exposure time (min)	360.0	350.5 ± 16.7
	Frequency/hour	0	17.3 ± 3.8
	Mean	67.8 ± 11.9	71.7 ± 11.6
Heart rate (BPM)	Maximum	116.0 ± 16.6	120.5 ± 9.2*
	Mean	96.8 ± 1.3	90.2 ± 1.1*
SpO_2 (%)	Maximum	98.1 ± 0.4	98.4 ± 0.5
	Minimum	93.2 ± 3.9	64.3 ± 5.9*
	≤90 %	0	124.1 ± 31.6
Time SpO_2 (minutes)	≤85 %	0	50.8 ± 14.5
	≤80 %	0	25.8 ± 7.9

Datas are mean ± standard deviation

* Statistical difference between normoxia and intermittent hypoxia ($p < 0.05$)

After a peak at 30 min, insulin levels declined more steeply during intermittent hypoxia sessions (condition × time interaction, $p < 0.05$) (Fig. 2d). Regardless of time, NEFA levels were significantly higher during intermittent hypoxia sessions (condition effect, $p < 0.05$) (Fig. 2e). No difference in circulating epinephrine and norepinephrine concentrations were observed between experimental conditions (data not shown).

Subcutaneous adipose tissue metabolism

Adipose tissue LPL activity (Fig. 3a) and ANGPTL4 expression (Fig. 3b) were affected neither by the meal nor the experimental conditions. Adipose tissue MT3 gene expression levels remain comparable before and after the meal in normoxia, but increased 4-fold under intermittent hypoxia. This interaction fell short of statistical significance (condition × time interaction, $p = 0.1$) (Fig. 3c).

Basal and stimulated lipolytic rate assessed from isolated subcutaneous abdominal adipocytes before and 3 h after the meal are presented in Fig. 4. A trend toward lower basal lipolytic rate in the postprandial phase compared to baseline measurements was observed in both conditions (effect of time, $p = 0.1$, Fig. 4a). Adenosine deaminase (ADA)-stimulated lipolysis was significantly and similarly reduced postprandially compared to baseline in both conditions (effect of time, $p < 0.05$) (data not shown). The dose-dependent lipolytic responses to isoproterenol (β-adrenoceptor [AR] agonist) were significantly and similarly reduced postprandially in both conditions (effect of concentration, $p < 0.01$) (Fig. 4b). Neither the meal nor the conditions affected the antilipolytic effects of epinephrine (mixed α2/β-AR agonist) and UK- 14304 (α2- AR agonist) (effect of concentration, $p < 0.001$) (Fig. 4c, d).

Discussion

Using differentiated human preadipocytes and subcutaneous abdominal adipose tissue biopsies from healthy individuals, we investigated the effects of acute hypoxia on adipose tissue lipid storage and/or mobilization functions. We show that 24 h of hypoxia significantly inhibits the activity of a key enzyme involved in adipose tissue TG deposition, LPL, in differentiated human preadipocytes. To explore whether the inhibitory effect of hypoxia on adipose tissue functions are noticeable in humans, young, healthy men were exposed for 6 h to acute intermittent hypoxia, an experimental model that has been proposed to study the metabolic effects of OSA. Acute exposure to intermittent hypoxia was sufficient to alter postprandial NEFA levels, as well as glucose and insulin levels, but did not alter circulating triglycerides nor subcutaneous adipose tissue lipid storage and/or mobilization functions.

Effects of hypoxia on LPL activity in differentiated human preadipocytes

To our knowledge, this is the first study examining the effects of hypoxia on LPL activity in differentiated human subcutaneous abdominal preadipocytes. Our results show a 6-fold reduction in LPL after a 24 h-incubation in hypoxic conditions. Consistently, ANGPTL4, a major post-translational regulator of LPL activity which inactivates LPL at the plasma membrane of adipocytes [20], was significantly increased after hypoxia, as previously reported by Wood et al. [21]. These observations confirm that the potential for lipid uptake of differentiated human preadipocytes is sensitive to an acute decrease in oxygen availability. It also complements recent evidence indicating that hypoxia impedes expression level of genes involved in de novo lipogenesis in human visceral adipose tissue [22].

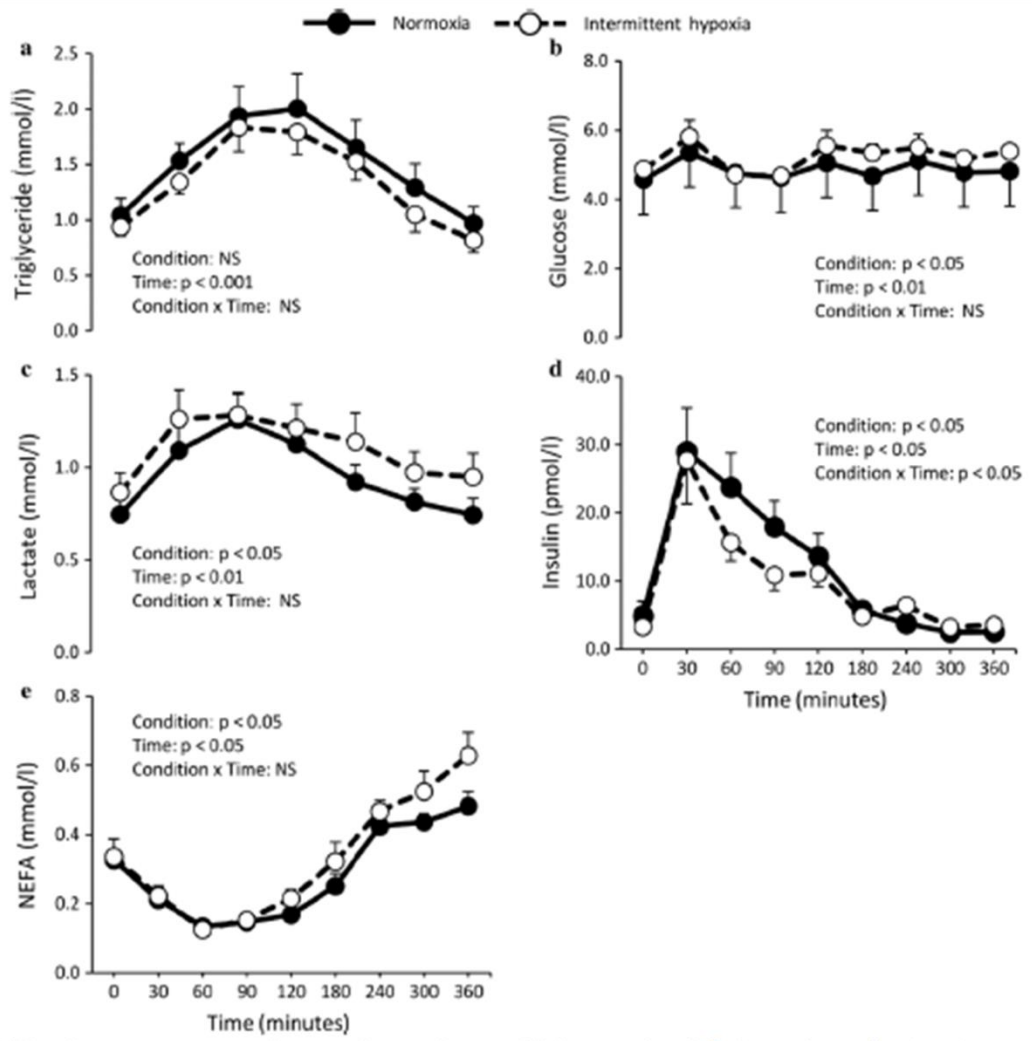

Fig. 2 Effect of normoxia or intermittent hypoxia on fasting and postprandial plasma **a** triglyceride, **b** glucose, **c** lactate, **d** insulin and **e** non-esterified fatty acids (NEFA) levels in healthy men. Values are mean ± standard error. *NS* not significant

Metabolic (non-lipid) effects of intermittent hypoxia in humans

In order to determine whether the reduction in LPL activity, observed in differentiated preadipocytes exposed to hypoxia, is translated in vivo, 10 young, healthy men were exposed to intermittent hypoxia in the postprandial state. Intermittent hypoxia was chosen over chronic hypoxia based on its similarity to sleep apnea, a disorder that is associated with an altered lipid profile [3, 23]. A fat-rich meal was also given to our participants based on numerous animal studies suggesting that postprandial triglyceride clearance is impaired by hypoxia [8, 10]. Our experimental setup clearly induced a systemic response: besides oxyhemoglobin desaturation cycles (by design), heart rate sharply and systematically increased during hypoxic cycles, reflecting a hypoxia-induced increase in sympathetic tone. As compared to values observed in normoxia condition, glucose and lactate levels were significantly increased after 90 min of intermittent hypoxia exposure, likely reflecting a shift in energy substrate utilization. Any changes in energy substrate partitioning, however, were impossible to confirm by indirect calorimetry, due to the constant changes in inspired and expired gas mixture.

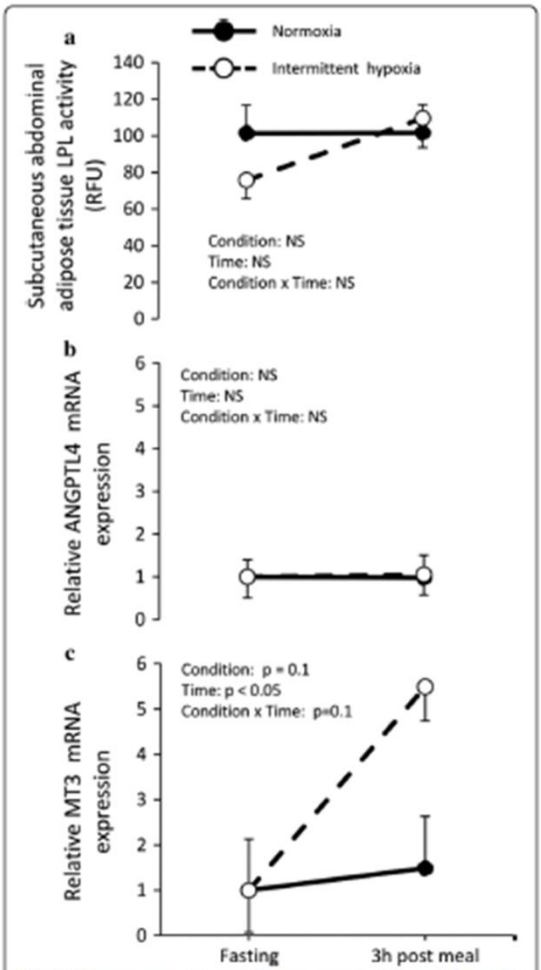

Fig. 3 Subcutaneous adipose tissue **a** lipoprotein lipase (LPL) activity, **b** angiopoietin-like 4 (ANGPTL4) gene expression and **c** metallothionein-3 (MT3) gene expression measured before (fasting) and 3 h post meal under normoxia and intermittent hypoxia in healthy men. Values are mean ± standard error. *NS* not significant

Effects of intermittent hypoxia on lipid and adipose tissue metabolism

No significant difference in postprandial triglyceridemia excursion was observed during intermittent hypoxia. Consistently, postprandial LPL activity, measured from adipose tissue biopsies, was not different between normoxia and intermittent hypoxia conditions. Despite a 4-fold increase in abdominal subcutaneous adipose tissue MT3 expression, which likely suggests that adipose tissue have been exposed to reduced partial pressure in oxygen, ANGPTL4 expression was not induced by the intermittent hypoxia session. The absence of changes in LPL activity and ANGPTL4 expression suggests that the clearance rate of TG by adipose tissue was likely not affected by intermittent hypoxia in our study sample. These results are not consistent with those from animal studies (mice) demonstrating that acute exposure to hypoxia [10] or chronic intermittent hypoxia [8, 9] delays plasma TG clearance and decrease subcutaneous LPL activity in white adipose tissue following a meal. These discrepancies, if not species-related, may be explained by the severity of the hypoxic stress. While the current study was conducted with intermittent hypoxia at a rate of 17.3 ± 3.8 events/hour for 6 h, Drager et al. [9] conducted their animal studies with a frequency of 60 hypoxic events/hour and Jun et al. [10] used constant hypoxia for 6 h.

The slight but statistically significant increase in plasma NEFA after 120 min of intermittent hypoxia is in line with several past observations of increased NEFA in animals exposed to hypoxic conditions [10]. This is typically explained by an increase in sympathetic tone, which stimulates adipose tissue lipolysis [10]. Results of lipolytic responses in isolated adipocytes from adipose tissue biopsies suggest, however, that if an increase in lipolysis rate occurred in vivo, it did not translate into an altered ex vivo response to lipolysis stimulating/inhibiting agents. Instead, it appears that the meal provided to our participants had a clear inhibiting impact on the adipocyte lipolytic activity. To the best of our knowledge, this is the first study to report ex vivo lipolytic response in adipocytes before and after the consumption of a meal. Our observations clearly support a strong suppression of NEFA release by isolated adipocyte of lean individuals in the postprandial phase. It is important to note, however, that despite the clear postprandial inhibition of lipolysis, adipocytes were still responsive to epinephrine and isoproterenol. Accordingly, the elevated plasma NEFA levels observed during intermittent hypoxia could still come from an increase in sympathetic drive, which should have been less present in the normoxia session. Other contributing factors to the increase in plasma NEFA during the intermittent hypoxia session include an earlier relief of lipolysis inhibition by insulin, and/or a decrease in circulating fatty acid utilisation by peripheral organs, leading to their accumulation in circulation. An increase in NEFA levels, in the long term, could lead to an increase in concentration of very low-density lipoprotein, small dense low-density lipoprotein particles, and elevated apolipoprotein B concentrations in plasma, all of which are associated with increased risk of coronary heart disease and stroke [24].

Some limitations of this study warrant discussion. First, in our in vitro experiments, only two different

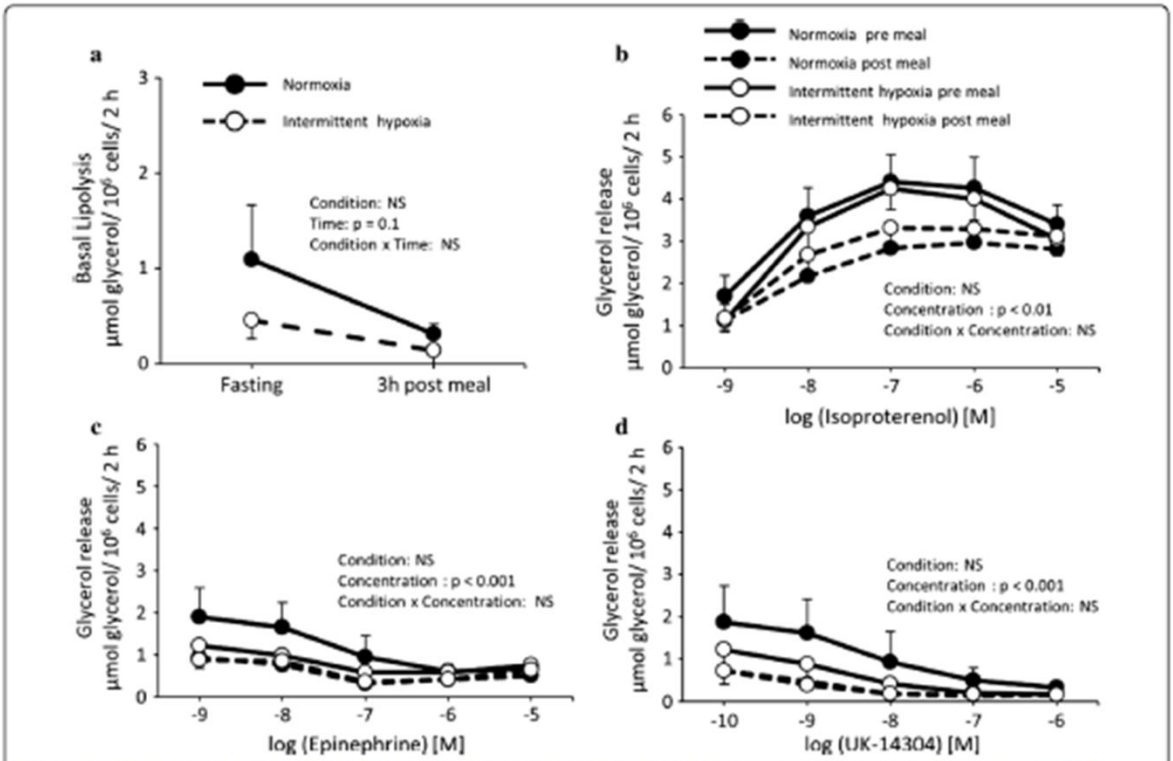

Fig. 4 **a** Basal lipolytic rate as well as effect of **b** isoproterenol (β-adrenoreceptor (AR) agonist), **c** epinephrine (mixed α2/β-AR agonist) and **d** UK-14304 (α2- AR agonist) on lipolysis in subcutaneous abdominal isolated adipocytes of healthy men before and 3 h after a meal under normoxia and intermittent hypoxia. Values are mean ± standard error. *NS* not significant

oxygen concentrations were tested: 3 % and 21 % O_2. Since it has been reported that adipocytes are sensitive to even relatively small changes in oxygen level within the physiological range [12, 25], further studies with different concentrations of oxygen could be undertaken. Limitations of our in vivo studies includes: the duration of intermittent hypoxia, which was brief and limited to only 6 h in order to limit burden and potential side-effects on the hypoxia-naïve participants; the severity of the intermittent hypoxia, which was equivalent to moderate OSA; and the homogeneity of our study sample, which consisted exclusively of healthy young men [2]. All these limitations limit the generalization of our metabolic observation to individuals suffering from OSA. OSA patients are likely exposed to intermittent hypoxia on a daily basis, and a large proportion of them exhibit metabolic complications [26]—increased adiposity, dyslipidemia, and insulin resistance (consequently of OSA or not)—that may synergistically exacerbate the negative lipid-altering effects of intermittent hypoxia. Finally, adipose tissue LPL activity is both sex and depot sensitive [14]. One could argue that these confounding factors may explain part of the discrepancy between our in vitro and in vivo observations since preadipocytes were obtained from female donors, while our in vivo experiments included only male subjects. While it is possible that sex and depot can affect adipocytes responses to hypoxia, it should be emphasized that our in vitro approach served only as a proof of concept that differentiated human fat cells, regardless of the donor's sex or adipose tissue depot, show a reduction in LPL activity under hypoxia. Regarding our choice of sampling site, the periumbilical region was chosen because the subcutaneous abdominal adipose tissue is responsible for most (45–50 %) of the clearance of exogenous lipids in humans [27, 28]. The remaining of the postprandial triglyceride clearance is proposed to be LPL-mediated in various other sites such as the subcutaneous femoral and visceral adipose tissues as well as the heart. Future studies remain to be performed to investigate how these other sources of LPL activity could be affected by intermittent hypoxia and to examine whether intermittent hypoxia affects the various sources of LPL similarly in men and women.

Conclusions

Our in vitro results indicate that hypoxia significantly inhibits lipoprotein lipase activity in differentiated human preadipocytes, while in vivo observations show that an acute session of intermittent hypoxia significantly increases postprandial NEFA levels, but not postprandial circulating TG, adipose tissue LPL activity, or adipocyte lipolysis, in healthy young men.

Authors' contributions

All authors had full access to all of the data in the study and gave final approval of the submitted version. Study design and conduct: PI, JFM, EC and BM. Data collection and analysis: JFM, EC, PI and BM. Data interpretation: PI, JFM, BM. Manuscript writing: BM, JFM and PI. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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Appendix C: List of published abstracts during PhD tenure

1. **Mahat B**, Mauger J, Imbeault P. Effects of different oxygen concentrations on human differentiated preadipocytes lipogenic and lipolytic functions. Experimental Biology. USA. April 22-26, 2017; 31: suppl.700.2.

2. Imbeault P**,** Chassé É**, Mahat B**, Clare L, Mauger J. The effect of acute exposure to normobaric hypoxia on postprandial triglyceride levels. The 20th International Hypoxia Symposium, Canada. February 7-12, 2017; W35, page 27.

3. Pépin A, Chassé É, **Mahat B**, Mauger J, Imbeault P. Modulation of appetite levels during acute intermittent hypoxia without changes in plasma leptin concentrations in humans. Canadian Nutrition Society, Canada. 2016; 41: S34.

4. **Mahat B**, Chassé E, Mauger J, Imbeault P. Effects of acute hypoxia on human adipose tissue lipoprotein lipase activity and lipolysis. Experimental Biology. USA. April 2-6, 2016; 30: suppl. 758.6.

5. **Mahat B**, Chassé E, Mauger J, Imbeault P. The effect of acute intermittent hypoxia, a simulating model of obstructive sleep apnea, on adipose tissue lipolysis in healthy humans. 4th National Obesity Summit, Canada. April 28- May 2, 2015; 39; suppl. 1, page S49.

6. Chassé E, **Mahat B**, Mauger J, Imbeault P. The effects of intermittent hypoxia, a simulating model of obstructive sleep apnea, on lipid levels in healthy humans. 4th National Obesity Summit, Canada. April 28- May 2, 2015; 39; suppl. 1, page S45.

7. **Mahat B,** Chassé E, Ait-Ouali S, Mauger J, Imbeault P. The effect of acute intermittent hypoxia, a simulating model of obstructive sleep apnea, on triglyceride levels in humans. 4th Canadian Student Obesity Meeting, Canada. June 18- 21, 2014; 52: P-8C.

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