The effects of BPA and BPS on skeletal muscle and adipose tissue metabolism

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Abstract

Background. Bisphenol A (BPA) and BPS are environmental pollutants that are associated with the development of insulin resistance and type 2 diabetes (T2D). Although skeletal muscle and adipose tissue dysfunction are involved the development of insulin resistance, there are few studies that have investigated the effects of bisphenols on their metabolism. In this study, we investigated the effects of BPA and BPS exposure on skeletal muscle and adipose tissue metabolism to determine how they contribute to the development of T2D.

Methods. L6 muscle cells were treated with BPA during the last 24 hours of differentiation, and mitochondrial function and glucose metabolism was measured. Human subcutaneous adipose tissue was incubated for 24 or 72 hours with BPA or BPS, and adipokine gene expression and glucose metabolism was measured in adipose tissue.

Results. L6 muscle cells treated with high concentrations of BPA (10^5 nM) had mitochondrial dysfunction and a compensatory increase in glucose metabolism; however, there were no effects at environmentally-relevant concentrations. Adipose tissue treated with BPA for 24 hours had reduced expression of proinflammatory cytokines and adipokines, and reduced insulin-stimulated glucose uptake.

Conclusions. BPA exposure for 24 hours did not alter L6 muscle cell mitochondrial function and glucose metabolism at environmentally-relevant concentrations; however, adipose tissue had altered proinflammatory expression and glucose metabolism at low concentrations. This has important implications in regulatory guidelines in the use of BPA in the manufacturing of consumer products.

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Abbreviation List

AdipoR- Adiponectin receptors

Akt- Protein kinase B

AMPK- 5′ AMP-activated protein kinase

aP2- Adipocyte P2

ASC- Adipose derived stromal cells

BAT- Brown adipose tissue

Bcl-2 - B-cell lymphoma 2

BPA- Phytoestrogens, bisphenol A

BPS- Bisphenol S

CEBPA- CCAAT enhancer-binding protein alpha

CREB- cAMP response element-binding protein

DAG- Diacylglycerides

DDT- Dichlorodiphenyl-trichloroethane

E2- 17β-estradiol

EDC- Endocrine-disrupting chemical

ER- Estrogen receptor

ERE- Estrogen response elements

ERK- Extracellular signal-regulated kinases

ERRϒ- Estrogen-related receptor ϒ

ETC- Electron transport chain

FA- Fatty acids

FABP4- Fatty acid-binding protein 4

FFA- Free fatty acids

GLUT1- Glucose transporter 1

GLUT4- Glucose transporter 4

GPR30- G protein-coupled receptor 30

GPx- Glutathione peroxidases

Grx- Glutaredoxins

GSK3- Glycogen synthase kinase 3

IGF- Insulin growth factor

IGFB-1- Insulin growth factor binding protein

IL-6- Interleukin-6

IL-10- Interleukin-10

IRS1- Insulin receptor substrate 1

IRS2- Insulin receptor substrate 2

LDR- Ligand binding domain

LOAEL- Lowest observed adverse effect level

LPL- Lipoprotein lipase

MAPK- Mitogen-activated protein kinase

mER- Membrane ER

MSC- Mesenchymal stem cell

Mfn2- Mitofusin 2

NHANES- National Health and Nutrition Examination Survey

OXPHOS- Oxidative phosphorylation of ADP

PCB- Polychlorinated biphenyl

PFOA- Perfluorooctanoic acid

PI3K- Phosphoinositol 3-kinase

PKC- Protein kinase C

POP- Persistent organic pollutants

PGC1- Peroxisome proliferator-activated receptor gamma coactivator 1-alpha

PPARγ- Peroxisome proliferator-activated receptor

ROS- Reactive oxygen species

S6K- S6 kinase

Ser 307- Serine 307

SERM- Selective ER modulator

SOCS3- Suppressor of cytokine signaling 3

SOD- Superoxide dismutase

SREBF1- Sterol regulatory element-binding protein 1

SVF- Stromal vascular cell

T1D-Type 1 Diabetes

T2D- Type 2 Diabetes

TBC1D1-TBC1 domain family member 1

TGs- Triglycerides

TGFβ- Transforming growth factor β

Thr308- Threonine 308

TNF-α- Tumor necrosis factor-α

TZD- Thiazolidinedione

UCP- Uncoupling proteins

UGT- UDP-glucuronosyltransferases

UGT2B1- UDP-glucuronosyltransferase 2B1

WAT- White adipose tissue

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1. Introduction

1.1 Diabetes

1.1.1 Prevalence of diabetes

As of 2014, it is estimated that there are 422 million adults living with diabetes worldwide, as the global prevalence has nearly doubled since the 1980's (World Health Organization, 2016). This highlights increasing risk factors over the last few decades. In 2017, 7.5% of Canadians over the age of 12 were reported to be diagnosed with diabetes, and those who were overweight or obese were more likely to report being diabetic (Statistics Canada, 2017). In Canada, approximately 90% of diabetes cases are type 2 diabetes (T2D), 9% are type 1 diabetes (T1D), and the remaining 1 % are other types of diabetes (Public Health Agency Canada, 2017). Furthermore, the prevalence of diagnosed diabetes increases with age, likely due to increasing risk factors (Public Health Agency Canada, 2017). Poorly managed diabetes can lead to other health complications, such as cardiovascular disease, kidney failure, nerve damage, and limb amputations (World Health Organization, 2016). For example, in the United States, 75% of individuals with diabetes have concomitant hypertension (Long & Dagogo-Jack, 2011). Comorbidity between diabetes and depression is also a major health concern, as it is unrecognized or treated in two thirds of patients (Katon, 2008). Furthermore, depression can lead to nonadherence to diabetes self-care, therefore, resulting in worse clinical outcomes (Katon, 2008). Both diabetes and its complications can have a negative economic impact. Economic loss can range from individuals with diabetes that face high medical costs or loss of employment, or the national economy due to medical costs and sustaining a health care system (World Health Organization, 2016). In 2013, approximately 208 billion dollars was spent in Europe and 350 billion dollars was spent in North America and the

Carribbeans on diabetes related healthcare (Ley et. al, 2014). Together, the health and economic burden of diabetes highlights the importance of prevention and management of this disease.

1.1.2 What is diabetes?

Diabetes is a chronic disease that is characterized by lack of insulin production by the pancreatic β-cells and/or the inability of insulin target tissues and cells to appropriately respond to insulin (World Health Organization, 2016). Glucose levels in the circulation is tightly regulated by the hormones insulin and glucagon (Röder, Wu, Liu and Han, 2016). In healthy individuals, increased levels of blood glucose is followed by insulin secretion into the circulation by pancreatic β cells leading to glucose transport into tissues (Röder, Wu, Liu and Han, 2016). Contrarily, when blood glucose levels are low, α cells release glucagon, which increases blood glucose by stimulating gluconeogenesis and glycogenolysis (Röder, Wu, Liu and Han, 2016). Reduced levels of circulating insulin and/or reduced response to insulin can lead to persistent hyperglycemia, which is the definition of diabetes (McDonnell and Umpierrez, 2012). Diabetes is diagnosed when fasting plasma glucose is ≥ 7.0 mmol/L (126 mg/dL), plasma glucose is ≥ 11.1 mmol/L (200 mg/dL) following an oral glucose tolerance test, or glycated hemoglobin is ≥ 48 mmol/mol (≥ 6.5 %) (Ghigo, Porta and Matschinsky, 2014).

1.1.3 Glucose homeostasis

The concentration of plasma glucose is dependent on the rate of glucose entering and exiting the circulation (Aronoff et al., 2004). Sources of glucose include intestinal absorption, gluconeogenesis, and glycogenolysis (Aronoff et al., 2004). Gluconeogenesis and glycogenolysis are under the regulation of glucagon, a hormone produced by the α cells of the pancreas, during periods of fasting (Aronoff et al., 2004). Increased blood glucose and amino acids following

ingestion of a meal are regulated by insulin, a peptide hormone produced by β cells of pancreatic islets, by stimulating glucose uptake and glycogen synthesis in cells (Aronoff et al., 2004). Furthermore, insulin inhibits the release of glucagon from α cells, therefore, reducing overall blood glucose levels (Aronoff et al., 2004). Insulin resistance is a condition that is characterized by reduced response to insulin and subsequent transport of glucose into cells (Ormazabal et al., 2018). Early stages of insulin resistance often lead to increased secretion of insulin from β cells to compensate for lack of glucose transport into cells (Cavaghan, Ehrmann and Polonsky, 2000). Eventually, chronic insulin release can lead to impairments to β cell function and a subsequent reduction in insulin secretion (Cavaghan, Ehrmann and Polonsky, 2000).

1.1.4 Causes of diabetes

The most common forms of diabetes can be categorized into three main types, T1D, T2D, and gestational diabetes. Type 1 diabetes is marked by deficient insulin-production by the pancreas, whereas T2D is often the result of ineffective usage of insulin produced by the body. Gestational diabetes is the onset of insulin resistance and hyperglycemia during pregnancy (World Health Organization, 2016).

The exact causes of T1D are not completely understood. Currently, it is believed that T1D is the result of genetic and environmental factors. In T1D, it is thought that the immune system attacks pancreatic β cells, leading to insufficient insulin production and release (Kahanovitz, Sluss and Russell, 2017). Due to this, T1D is sometimes referred to as insulin dependent diabetes.

The exact causes of gestational diabetes are unknown, however, it is believed that hormones released from the placenta induce insulin resistance in peripheral tissues (Poulakos et al., 2015). This leads to increased blood glucose and followed by chronic increased insulin release from the

pancreas (Poulakos et al., 2015). Gestational diabetes is often resolved after the baby is born, but mothers who had gestational diabetes and the babies themselves are at higher risk to develop T2D in the future (Poulakos et al., 2015).

T2D accounts for the majority of diabetes cases in the world (World Health Organization, 2016). T2D has been shown to be the result of many different factors, such as genetics, diet, obesity, lack of physical activity or increased sedentarity, and environmental contaminants (World Health Organization, 2016). There is some evidence that development of T2D has a genetic component. For example, the concordance of diabetes in monozygotic twins is 70%, whereas it is 20-30% in dizygotic twins (Kaprio et al., 1992). Furthermore, studies have shown that individuals with a family history of T2D are at an increased risk for the development of T2D, as they have reduced insulin sensitivity, insulin clearance, and insulin growth factor (IGF) binding protein (IGFB-1) (Arslanian et al., 2004). Over the last few decades, urbanization and environmental transitions have led to changes in our physical activity, food production, and food processing (Ley, Hamdy, Mohan and Hu, 2014). Improvements in technology, shifts in energy expenditure at work, and improved transportation, have led to an increase in a sedentary lifestyle and a rise in obesity, which is linked to the development of T2D (Popkin, Adair and Ng, 2012). In addition, fat distribution is also an important factor in insulin resistance development. For example, individuals with more peripheral fat are more insulin sensitive than those with central obesity (Karpe, Dickmann and Frayn, 2011). Over the last few decades, the T2D epidemic has been greatly focused on carbohydrate consumption and physical activities. Interestingly, the start of this epidemic is also positively associated with increased production of chemical pollutants (Pizzorno, 2016). It has been shown that there is a synergistic association between body load of persistent organic pollutants (POPs) and the development of metabolic syndrome (Pizzorno, 2016). It is hypothesized

that in addition to traditional risk factors, increased production and exposure to environmental contaminants can contribute and accelerate the development of T2D (Pizzorno, 2016).

1.1.6 Environmental contaminants and type 2 diabetes

Diabetes is a multifactorial disease, caused by a complex interplay of genetic and environmental factors (Murea, Ma and Freedman, 2012). Determining which factors in our environment contribute to altered glucose metabolism and insulin resistance is important in reducing the risk of developing T2D. Obesogens are chemical compounds that disrupt normal lipid metabolism, and can lead to obesity (Janesick and Blumberg, 2016). Environmental pollutants such as certain bisphenols are examples of obesogens and are considered a risk factor for the development of T2D (Maradonna and Carnevali, 2018). Other studies have shown that there is a strong dose-response relationship between serum concentration of POPs and diabetes prevalence (Lee and Jacobs, 2006). For example, when POPs and the development of T2D were compared, individuals in the top 10% of serum trans-nonachlor levels had a 12-fold increased risk (Pizzorno, 2016). In another study, serum levels of perfluorooctanoic acid (PFOA) was associated with the development of gestational diabetes, with each increment in standard deviation increasing the risk two-fold (Zhang et al., 2015). Some obesogens are considered to be endocrine disrupting chemicals (EDCs) (Pereira-Fernandes et al., 2013). Those chemicals are hormonally active compounds that mimic, block, or interfere with hormones in the body's endocrine system (Endocrine Disrupting Chemicals, Endocrine Society, 2020). They include chemical substances that occur naturally or are man-made and found in a variety of materials such as plastics, pesticides, and personal care products (World Health Organization, 2011), and examples include bisphenols, biphenyls, and plant phytoestrogens (Diamanti-Kandarakis et al., 2009). Route of exposure of EDCs mainly includes inhalation, ingestion, and dermal absorption (Rudel and Perovich, 2009). Endocrine

disrupting chemicals generally function by activation or repression of genes, alteration of hormone synthesis and transport, and epigenetic modifications (Diamanti-Kandarakis et al., 2009). Studies in the last few decades have demonstrated a link between EDCs exposure and the development of diabetes, either directly following exposure or intergenerationally (indirectly) (Chianese et al., 2017). Some EDCs are lipophilic, such as bisphenols, polychlorinated biphenyls (PCBs), and dioxins, and can be stored in adipose tissue and accumulate in the food chain (Lind and Lind, 2018). Although the diabetes epidemic is often attributed to carbohydrate consumption and lack of physical activity, the role of environmental toxins have been underestimated. In one review, Pizzorno (2016) describes the inconsistency between the start of the diabetes epidemic and increased carbohydrate consumptions. Instead, there are clear trends between increased diabetes prevalence and pollutant exposure (Lind and Lind, 2018). Understanding how certain environmental chemicals can contribute to the development of diabetes is important in improving the manufacturing regulation and thus exposure to these chemicals.

1.2 Bisphenols (BP's)

1.2.1 Xenoestrogens

Xenoestrogens are a subtype of EDCs that are manmade or from other sources such as plants that have estrogenic activity (Gregoraszczuk and Ptak, 2013). Exposure to xenoestrogens can lead to an overall increase in estrogen levels and can alter estrogen signalling cascades (Watson, Jeng and Guptarak, 2011). Examples of xenoestrogens include PCB, dichlorodiphenyl-trichloroethane (DDT), phytoestrogens, bisphenol A (BPA, a plastics additive) and phthalates (Gregoraszczuk and Ptak, 2013). Xenoestrogens exert their mechanism mainly through binding to estrogen receptor (ER)-α, ER-β, estrogen-related receptor γ (ERR-γ), and G protein-coupled receptor 30 (GPR30),

integrating genomic and non-genomic pathways to exert estrogenic effects (Watson, Jeng and Guptarak, 2011). There is tissue specific expression of the various estrogen receptors. For examples, in adipose tissue, there are high levels of ER-α, playing an important role in maintaining adipose tissue function and protecting against inflammation, whereas, in neurons ER-β and GPR30 are abundant, and may play a role in oxytocin release (Blüher, 2013; Xu et al., 2009; Adkins-Regan et al., 2014). Furthermore, xenoestrogens have also been shown to alter the intracellular levels of ER both at the gene and protein level, which can lead to metabolic defects (La Rosa et al., 2014). The binding of xenoestrogens to ER and altering the ratio of estrogen receptors has been implicated in several diseases (Jeng, Kochukov and Watson, 2010). For instance, BPA has been shown to upregulate ER-α, which is an important characteristic in the development of breast cancer (La Rosa et al., 2014).

1.2.2 Purpose and chemical properties of bisphenols

Bisphenols are a group of chemical compounds first synthesized in 1891 that are characterized by two hydroxyphenol groups (Ben-Jonathan and Hugo, 2016). In the mid 1930's, Edward Charles Dodd discovered the estrogenic effects of BPA just prior to discovering the synthetic estrogen diethylstilbestrol, a drug that is structurally similar to BPA, and used as a nonsteroidal estrogen to support pregnancies in women with recurrent miscarriages (Figure 1). Diethylstilbestrol has been largely discontinued following the discovery of its toxic effects, such as the increased risk of developing breast cancer and clear-cell carcinoma (Reed and Fenton, 2013). Bisphenol A (2,2-bis(4-hydroxyphenyl)propane) contains a dimethyl derivative and is soluble in organic solvents (Figure 1) (Ben-Jonathan and Hugo, 2016). Repeating BPA monomers are used to produce polycarbonate plastics and epoxy resins (Ben-Jonathan and Hugo, 2016). The carbonate linking of BPA monomers is often stable, however, BPA can leach due to incomplete polymerization or

degradation of polymers at high temperatures or altered acidity (Ben-Jonathan and Hugo, 2016). Furthermore, BPA is structurally similar to 17β-estradiol (E2) and has been shown to have hormone-mimicking properties (Figure 1). Since the 1950s, BPA has been produced in large quantities, due to its cost efficient method of producing lightweight, colorable, easy to mold products such as plastic bottles, food containers, thermal receipts, and dental sealants (Ben-Jonathan and Hugo, 2016). Early research showed low toxicity and rapid metabolism of BPA, which led to the safety approval of use in plastic production (Sajiki and Yonekubo, 2003). It was later found both *in vivo* and *in vitro* studies that BPA acts as an endocrine disruptor, leading to adverse effects such as increased prostate weight, growth of mammary glands, and postnatal development, therefore challenging previous beliefs that BPA was safe at low doses (Vogel, 2009). Due to increasing concerns of the negative effects of BPA on health over the last few decades, it has recently been replaced by an analog, known as bisphenol S (BPS). Bisphenol S (4,4'-sulfonylbisphenol), is structurally similar to BPA, and has two hydroxyphenol groups around a sulfonyl group (Figure 1) (Ben-Jonathan and Hugo, 2016). Polymers of BPS are known as polyethersulfones (Ben-Jonathan and Hugo, 2016). There is seldom research on the toxicological effects of BPS, and thus, there are less regulations on its use in consumer products. Interestingly, recent studies suggest that BPS may have a stronger endocrine disrupting effects than BPA (Vandenberg et al., 2009). For example, in one study, BPS had a stronger inhibitory effect on testosterone release than BPA (Niederberger, 2015). Another study demonstrated that BPS had a more potent effect on lipid metabolism in 3T3-L1 cells than BPA, such as adipogenesis and peroxisome proliferator-activated receptor (PPARγ) activation (Ahmed and Atlas, 2016). Therefore, further research is required to understand the effects of both BPA and its analog BPS in order to improve manufacturing regulations.

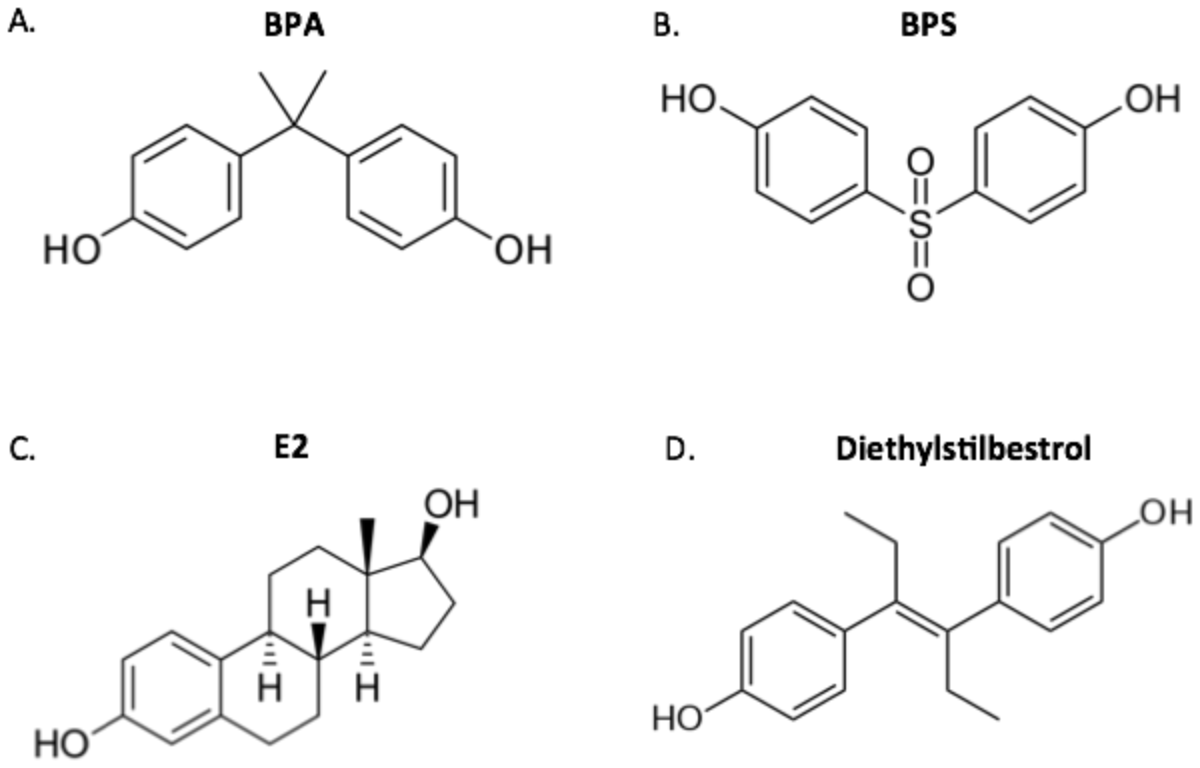

Figure 1. Chemical structure of BPA, BPS, 17β-estradiol (E2), and diethylstilbestrol. (A) BPA is a dimethyl derivative and consists of two hydroxyphenol groups. (B) BPS is a sulfonyl derivative and consists of two hydroxyphenol groups. (C) E2 is a sex hormone derived from cholesterol that contains a hydroxyphenol group that interacts with the estrogen receptor (ER). (D) Diethylstilbestrol is a synthetic estrogen that contains two hydroxyphenol groups.

1.2.3 Bisphenol exposure

BPA has been detected in 90% of urine samples of Canadians between the age of 3 to 79, according to the Canadian Health Measure Survey (Statistics Canada, 2015). They showed that men had higher levels of BPA in urine than women, and children and youth tended to exhibit higher levels than adults over the age of 20 (Statistics Canada, 2015). Furthermore, they found significantly higher concentrations of BPA in urine in individuals with obesity compared to those with normal weight or who were overweight (Statistics Canada, 2015). Similar trends demonstrated globally, such as in the NHANES results, where 93% of Americans had detectable BPA in their urine, which was considerably higher in children (Calafat et al., 2008). BPA is often considered a weak environmental estrogen, since several studies have shown BPA to be more than 1000 times less potent that estrogen (Vandenberg et al., 2009; Feldman M.D., 1997). Recent studies, however, have shown that BPA at low doses (as low as 1 pM) have similar efficacy as estrogen to elicit a metabolic response (Wozniak, Bulayeva and Watson, 2005). Levels of unconjugated BPA in blood, tissue, and urine has been shown to be higher than what is expected based on the relatively rapid metabolism of BPA (Domoradzki, 2003). In 1988, the Environmental Protection Agency stated that 50 μg/kg/day of BPA is considered safe (reference dose), which was considered to be 1000 times lower than the lowest observed adverse effect level (LOAEL) (Welshons, Nagel and vom Saal, 2006). Interestingly, however, BPA exposure in animal models have shown effects at lower than this dose. For example, pregnant mice administered 10 μg/kg/day of BPA had permanent changes in reproductive organs of male offsprings, whereas a dose of 0.25 μg/kg/day lead to altered mammary gland growth, postnatal growth, and rate of sexual maturation (Timms et al., 2005). Low concentrations of BPA often used in animal studies, are similar to the levels found in humans, based on exposure from plastic containers, cans, dental sealants, and even drinking

water, which are often in the low nanomolar range (Welshons, Nagel and vom Saal, 2006). For example, Le et al. (2008) measured the amount of BPA that leached from polycarbonate water bottles using sensitive and quantitative methods. They found that at room temperature 0.2 to 0.79 ng was transferred per hour, and boiling increased the rate by 55-fold (Le, Carlson, Chua and Belcher, 2008). Furthermore, they showed that BPA levels corresponding to levels in water were able to activate rapid non-genomic pathways in cerebellar neurons (Le, Carlson, Chua and Belcher, 2008). In another study, it was described that for humans with the lowest fifth percentile of pollutant exposure, the concentration of BPA found in serum, urine, and tissues, of about 0.1 μg/L, exceeded the adverse effect levels seen in many animal models (vom Saal and Welshons, 2006). Therefore, it is clear that the levels of BPA found in humans corresponds to low levels used in many experimental studies. Furthermore, due to a combination of these sources of BPA (ie. from different products), the concentrations in humans can be higher than predictions made by manufacturing industries (Gray et al., 2004). A summary of environmentally-relevant concentrations used in selected *in vivo* and *in vitro* studies are presented in Table 1. These can be compared to levels found in human serum, urine, and tissue samples found in Table 2.

Table 1. Summary of in vivo and in vitro studies using environmentally-relevant concentrations of BPA or BPS

Model	Dose	Treatment	Effect	Author(s)
In vivo				
CD-1 mice	BPA (0.025 and 0.25 μg/kg/bw)	Subcutaneous osmotic pump, daily	Changes in sexual dimorphic anatomical and behavioral	(Rubin et al., 2006)
Pregnant CF-1 mice	BPA (2.4 μg/kg/bw/day)	Oral (daily during day 11-17 of gestation)	↑ Female offspring body weight	(vom Saal et al., 2012)
Swiss albino OF1 mice	BPA (100 μg/kg/day)	Subcutaneous injection, twice daily, for 8 days	↑ Blood glucose ↑ Insulin signaling markers	(Batista et al., 2012)
Pregnant sheep	BPA or BPS (500 μg/kg/day)	Daily subcutaneous injection	Muscle fiber hypertrophy	(Jing, Pu, Gingrich and Veiga-Lopez, 2019)
OLETF rats	BPA (1 and 10 μg/kg/ day)	Once daily by oral gavage	Tended to reduce PPARγ ↑ Serum insulin	(Yang et al., 2014)
CD-1 mice	BPA (5, 10, or 40μg/kg)	Orally daily GD 11 to PND 8	Pubertal differences in the female progeny	(Ruiz-Pino et al., 2019)
Female C57BL/6 mice	BPS (50 μg/kg/day),	Oral daily (added to drinking water) for 10 weeks	↓ Glutathione metabolism	(Gao et al., 2020)
Pregnant wistar rats	BPS (10 and 50 μg/kg/day)	Oral daily between GD to PND 21	↓ Food intake Smaller fat droplets	(da Silva et al., 2019)

In vitro				
3T3-L1 adipocytes	0.1-3 nM (BPA)	Days 2 to 7 of differentiation	↑ Pro-inflammatory markers	(De Filippis, Li and Rosen 2018)
Fish primary macrophages	0.1-1000 µg/L	6 hours	↑ ROS and total antioxidant ability	(Qiu et al., 2018)
HepG2 cells	1 nM-10µM	24 hours	↑ Total cholesterol content	(Li et al., 2019)
Breast adipose explants (women)	0.1 and 1 nM (BPA)	6 hours incubation of adipose tissue	↓ Adiponectin	(Hugo et al., 2008)
Abdominal subcutaneous adipose tissue (mature adipocytes isolated)	0.1, 1, and 10 nM (BPA)	6 hours incubation of adipose tissue	↓ Adiponectin	(Hugo et al., 2008)

CF1: Carworth Farms strain 1, OF1: Oncins France 1, OLETF: Otsuka Long-Evans Tokushima Fatty, GD: gestational day, PND: postnatal day, C57BL/6: C57 black 6, ROS: reactive oxygen species.

Table 2. Levels of BPA and BPS found in human samples.

Endpoint	Levels measured	Authors
BPA		
Amniotic fluid	8.3 ng/mL (36 nM)	(Ikezuki et al., 2002)
Maternal serum	2.24 ng/mL (9.81 nM)	(Yamada et al., 2002)
Female serum (obese)	1.04 ng/mL (4.55 nM)	(Takeuchi et al., 2004)
Breast milk	0.61 ng/mL (2.67 nM)	(Sun et al., 2004)
BPS		
Male urine	0.36 ng/mL (1.38 nM)	(Jacobson et al., 2019)
Female urine	0.39 ng/mL (1.56 nM)	(Jacobson et al., 2019)
Maternal blood	0.11 ng/mL (0.44 nM)	(Zhang et al., 2020)
Cord blood	0.026 ng/mL (0.10 nM)	(Zhang et al., 2020)

1.2.5 Bisphenol metabolism

Following oral ingestion, bisphenols are absorbed in the gastrointestinal tract and metabolized in the liver into BPA-glucuronide by UDP-glucuronosyltransferases (UGT), and then excreted in the urine (Bushnik et al., 2010). BPA-glucuronide that reaches the intestinal tract can be reactivated by cleavage, primarily by bacterial enzymes, and can enter the bloodstream (VandeVoort et al., 2016). UDP-glucuronosyltransferase 2B1 (UGT2B1), is a UDP-glucuronosyltransferases isoform that glucuronidates BPA, and has been shown to be reduced in pregnant women and absent in fetuses, and slowly appears after birth (Welshons, Nagel and vom Saal, 2006). Interestingly, fetuses and neonates have high potency of BPA due to the limited capacity of the liver to conjugate BPA to BPA-glucuronide (Schönfelder et al., 2002). Furthermore, there is evidence of bioaccumulation of BPA during pregnancy, which does not occur in nonpregnant females, or males (Schönfelder et al., 2002). Therefore, pregnant women, fetuses, and newborns may have higher levels of metabolically active BPA in circulation due to lack of excretion (Welshons, Nagel and vom Saal, 2006). BPA and BPS are rapidly metabolised, as BPA has a half life of less than 6 hours, while BPS has a half life of less than 7 hours (Bushnik et al., 2010). Free BPA is considered active, however, due to the effective liver metabolism of BPA, it is believed that low levels of free BPA from oral ingestion is found in the circulation (European Commission-Scientific Committee on Food, 2002). According to the Scientific Committee on Food, the maximum amount of BPA in blood following oral ingestion is 2-8%, compared to 27-51% following intraperitoneal injection, or 65-76% following subcutaneous injection (European Commission-Scientific Committee on Food, 2002). Moreover, BPA-glucuronide has not been shown to have estrogenic activity (Welshons, Nagel and vom Saal, 2006). In males, it is believed that endogenous steroids alter BPA metabolism, which leads to higher circulating BPA (Takeuchi and Tsutsumi, 2002).

1.2.6 Molecular mechanism of bisphenols

Estrogen receptors (ER) are a group of proteins that are widely expressed in several cells, such as the breast cells, myocytes, and adipocytes. As mentioned previously, there are three known ERs that regulate all estrogenic effects: ER-α, ER-β, and GPR30 (Marino, Galluzzo and Ascenzi, 2006). Estrogen signaling can be divided into genomic and non-genomic signaling cascades. Genomic signaling involves the binding of E2 to ER-α or ER-β at the membrane or in the cytoplasm which is followed by translocation to the nucleus and binding to estrogen response elements (ERE) at or near the promoter of target genes (Marino, Galluzzo and Ascenzi, 2006). Non-genomic estrogen signaling is rapid and often involves membrane ER such as GPR30 and some membrane ER-α and ER-β (mER-α and mER-β) (Vrtačnik, Ostanek, Mencej-Bedrač and Marc, 2014). Binding of E2 to GPR30, mER-α, or mER-β can lead to activation of protein kinases and downstream phosphorylation of transcription factors and proteins (Vrtačnik, Ostanek, Mencej-Bedrač and Marc, 2014). Furthermore, binding of E2 to GPR30 has been shown to influx of intracellular calcium (Ca^{2+}), activation of the phosphoinositol 3-kinase (PI3K) signaling pathway, and mitogen-activated protein kinase (MAPK) signaling pathway both of which are involved in cell cell growth, proliferation, and differentiation. One example of E2 non genomic signaling occurs in HepG2 cells (a human liver cancer cell line), where E2 induces a rapid, non genomic response that regulates the PI3K/Akt signal transduction pathway (Marino, Acconcia and Trentalance, 2003). Investigations of the global expression targets of ER has shown that nearly half of the target genes of ER are downregulated following the binding of E2 to the receptor (Frasor et al., 2003). This included genes that normally inhibit cell cycle, are pro-apoptotic, and/or growth factors that inhibit proliferation, which aligns with other studies that have shown that E2 binding is linked to cell survival (Welboren, Stunnenberg, Sweep and Span, 2007). Interestingly, however, other studies

have shown that E2 can also be linked to apoptosis. For example, E2 leads to apoptosis in cells that have had long-term estrogen deprivation (Lewis-Wambi and Jordan, 2009). In addition, E2 has been shown to bind to ERRγ, which is a nuclear orphan receptor that acts as a constitutive activator of transcription (Kubo et al., 2009). This estrogen related receptor is expressed in a tissue specific manner, such as the adipose tissue, and skeletal muscle, and therefore, has tissue dependent action (Kubo et al., 2009). In adipose tissue, for example, activation of ERRγ is associated with adipocyte differentiation and alteration of adipocyte gene expression (Kubo et al., 2009). Thus, E2 can activate or suppress different signaling pathways genomically and non-genomically depending on the receptor it binds.

Research has shown that there is no single mechanism by which bisphenols exert their mechanism of action, however, proposed mechanisms include binding to the cytoplasmic ER α and β, mER α and β, and GPR30 (Ben-Jonathan and Hugo, 2016). Bisphenol A has the structural ability to bind ER α and β, however, at an affinity about 1000-2000 fold less than E2 (Acconcia, Pallottini and Marino, 2015). Following binding to the ER, this complex can translocate to the nucleus and can bind to the ER element and interact with co-activators and co-repressors to alter transcriptional activity (Acconcia, Pallottini and Marino, 2015). The displacement in the ligand binding domain (LDR) of the ER determines whether the ligand will act as an agonist or an antagonist. For example, in some instances BPA can bind to the LDR of ER-α in a similar manner as E2, and act as an agonist, however, may not have the same displacement when binding to ER-β, and thus acts as antagonist (Acconcia, Pallottini and Marino, 2015). Furthermore, BPA can bind to the small pool of ER-α and ER-β localized at the plasma membrane that can form multimolecular complexes and lead to rapid nongenomic responses, such as ER-α mediated PI3K/Akt pathways, or ER β-mediated MAPK signaling (Acconcia, Pallottini and Marino, 2015). There have been growing

studies that have demonstrated the effects of estradiol on nongenomic pathways, leading to a rapid and amplified response inside the cell, which can occur at very low concentrations. Binding of BPA to GPR30 is linked to altered oscillation of Ca^{2+}, which can lead to events such as endoplasmic reticulum stress, and insulin release (Acconcia, Pallottini and Marino, 2015). For example, 1 pM of BPA stimulates Ca^{2+} influx within 30 seconds in rat pituitary tumor cells (Wozniak, Bulayeva and Watson, 2005). Similarly, in mouse pancreatic β cells, 1 nM of BPA leads to phosphorylation of cAMP response element-binding protein (CREB) within 1 minute (Quesada et al., 2002). G protein-coupled receptor 30, which is now widely considered an ER, is expressed in a wide range of tissues, and BPA has been shown to bind to it with high affinity (Alonso-Magdalena et al., 2005). Therefore, it is clear that weak activity in one pathway does not elucidate potency in other pathways. Within the same tissue, there can be differences in estrogen-stimulated gene expression from different cells (Welshons, Nagel and vom Saal, 2006). For example, in one study BPA binding activity was measured in the uterine of rodents, using two methods: stimulation of uterine wet weight gain, or an estrogen-responsive reporter gene (Nagel, Hagelbarger and McDonnell, 2001). They showed that BPA was only a partial agonist for stimulating uterine wet weight gain, but was a strong agonist for reporter genes (targeting different cells) (Nagel, Hagelbarger and McDonnell, 2001). Furthermore, studies on animal models have shown that the dose required to elicit a response in one tissue can be different to the dose required for other tissues in the same animal (Morrissey, 1987). Bisphenol A has also been shown to bind ERRγ at low nanomolar doses with high affinity (Okada et al., 2008). In addition, Takeda et al. (2009) showed that BPA accumulates in the human placenta by binding ERRγ. Therefore, BPA is able to elicit its effects by binding the ER (α and β), GPR30 and ERRγ.

Bisphenol A has been shown to follow an oscillating non-monotonic dose response (Acconcia, Pallottini and Marino, 2015). A biphasic-U or inverted-U dose response was shown to be directly related to BPA concentrations (Acconcia, Pallottini and Marino, 2015). Therefore, bisphenols may not follow the conventional dogma that the higher the dose the greater the effect (Hill, Myers and Vandenberg, 2018). For examples, in one study low doses of BPA in rats were linked to the development of adenocarcinomas, however, this did not occur at higher doses (National Toxicology Program, 2018). Similarly, Hass et al. (2016) showed that mice treated with low doses of BPA had reduced sperm count, but adverse effects were not seen at higher concentrations. Pituitary cells exposed to BPA follow a U-shaped response 10^{-12}, 10^{-11}, and 10^{-8}M had an effect whereas 10^{-10} and 10^{-9} M did not (Wozniak, Bulayeva and Watson, 2005). Prior to 2012, the Environmental Protection Agency did not consider the non-monotonicity of BPA when determining regulatory standards (Hill, Myers and Vandenberg, 2018). The conventional dogma that there was always a linear dose response led to the assumption that if effects were not seen at high concentrations, effects would not be seen at lower concentrations. Therefore, understanding the effects of BPA at both low and high doses is crucial in modelling its non-monotonic dose effect and in turn forming appropriate regulations (Acconcia, Pallottini and Marino, 2015).

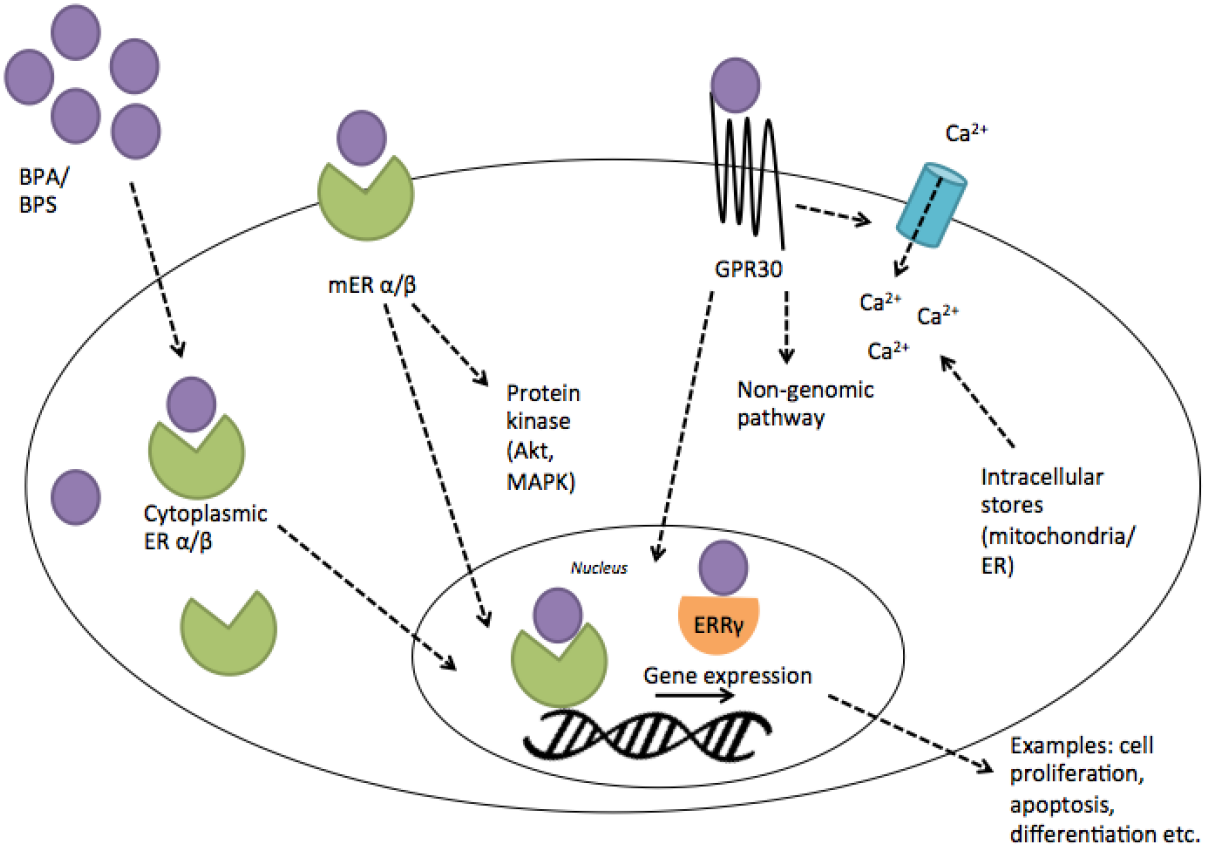

Figure 2. Molecular mechanism of bisphenols .BPA and BPS are able to exert their effects genomically or non-genomically. Following binding the membrane or cytoplasmic ER-α and ER-β, the receptor-ligand complex can translocate to the nucleus and bind to the gene promoter to alter gene expression. This genomic response can alter cell proliferation, apoptosis, and differentiation. BPA can also directly bind to ERRγ in the nucleus which can activate transcription of genes. Membrane ER-α and ER-β can also lead to non-genomic responses such as alter protein kinase activity. Bisphenols can also bind GPR30, which can lead to rapid non-genomic responses including influx in calcium. GPR30 is also able to exert its effects genomically.

1.2.7 Link between bisphenol A exposure and type 2 diabetes

In 2008, the Canadian Environment Protection Act classified BPA as toxic, raising awareness of the possible adverse effects of bisphenol exposure (Vogel, 2009). In the recent decades, there is a growing list of studies showing the link between BPA exposure and the development of T2D. For example, one study analyzed urine samples from the National Health and Nutrition Examination Survey (NHANES) and showed a positive association between high urinary BPA (>4.20 ng/ml) and T2D development, independent of diabetes risk factors (Shankar and Teppala, 2011).

Several studies have investigated the effects of BPA exposure on pancreatic function and glucose metabolism. Pancreatic β cells contain both ER-α and ER-β, therefore, are susceptible to the effects of BPA exposure. In one study, mice exposed to a single low dose (10 μg/kg) of BPA intraperitoneally showed increased insulin release followed by a rapid reduction in glycemia (Alonso-Magdalena et al., 2006). In the same study, they showed that long term BPA exposure in mice (intrapaneal twice daily for 4 days) lead to increased β-cell insulin content (the result of binding to the ER), hyperinsulinemia, and insulin resistance (Alonso-Magdalena et al., 2006). This is supported by *in vitro* studies showing that exposure of a mouse β cell line to BPA (100 ng/mL) for 1 hour leads to enhanced glucose stimulated insulin secretion (Makaji, Raha, Wade and Holloway, 2011). This was associated with mitochondrial stress and activation of B-cell lymphoma 2 (Bcl-2) family members and caspases which are responsible for apoptosis in pancreatic β cells (Makaji, Raha, Wade and Holloway, 2011). Thus, it is evident that BPA can induce β cell dysfunction and apoptosis, which in turn, can contribute to peripheral insulin resistance.

As previously mentioned, the liver is a major organ for the metabolism of xenobiotics, and BPA has been linked to liver toxicity. Mice treated with high doses of BPA (200 mg/kg body weight) have reduced hepatic glucose oxidation, insulin receptor expression, Akt phosphorylation, and glycogen production (Jayashree et al., 2013). Insulin resistance of the liver is a major contributor to T2D development, as reduced insulin response and uncontrolled glucose production by the liver can contribute to insulin resistance of peripheral tissues (Klover and Mooney, 2004). Furthermore, at low concentrations, HepG2 cells exposed to BPA (10 and 100 nM) for 2 hours show reduced oxygen consumption rate and ATP production, in addition to deteriorating mitochondrial architecture (Moon et al., 2012). This is important since mitochondrial dysfunction in the liver has been shown to be associated with liver and peripheral insulin resistance (ie. skeletal muscle insulin resistance) (Vianna et al., 2006). Therefore, BPA-induced alterations of hepatocyte metabolism can contribute to whole-body insulin resistance.

Although there is clear association between bisphenol exposure, insulin resistance and T2D, there are few studies that investigate the effects of bisphenols on skeletal muscle and adipose tissue metabolism. Both tissues play important roles in whole-body glucose homeostasis, therefore, understanding the effects of bisphenols on their metabolism would further elucidate the mechanisms bisphenols exert.

1.3 Skeletal muscle perturbations in type 2 diabetes

1.3.1 Insulin signaling pathway

Peripheral glucose uptake into the skeletal muscle can occur through different pathways; insulin-stimulated, contraction-stimulated, or through basal glucose uptake (Mackenzie and Elliott, 2014). Two important glucose transporters in the skeletal muscle are glucose transporter 1 (GLUT1) and

GLUT4. GLUT1 is responsible for basal glucose uptake in cells, whereas GLUT4 is an insulin or contraction-regulated glucose transporter.

Binding of insulin to the insulin receptor leads to tyrosine phosphorylation of the insulin receptor and subsequent phosphorylation of the insulin receptor substrate 1 and 2 (IRS1/IRS2) (Mackenzie and Elliott, 2014). This is followed by recruitment of PI3K and the phosphorylation of protein kinase B (Akt) at catalytic domain (Threonine/Thr308) and the carboxyl terminal hydrophobic domain (Serine/Ser473) (Mackenzie and Elliott, 2014). In the absence of insulin, the Akt substrate of 160 kDa (AS160, or TBC1D4) keeps Rab-GTPases inactive, thus keeping GLUT4 in a vesicle within the cytoplasm (Mackenzie and Elliott, 2014). Akt activation leads to the phosphorylation and inactivation of AS160, which in turn leads to GLUT4 translocation to the cell membrane and glucose uptake (Mackenzie and Elliott, 2014). Furthermore, Akt phosphorylates and inactivates glycogen synthase kinase 3 (GSK3). When active (i.e. dephosphorylated), GSK3 phosphorylates and inhibits glycogen synthase and thus glycogen synthesis (Mackenzie and Elliott, 2014). This leads to increased blood glucose or hyperglycemia. Therefore, inhibition of GSK3 leads to active glycogen synthase, increased glycogen synthesis, and reduced blood glucose.

Both *in vivo* and *ex vivo* studies have shown that contraction-stimulated glucose uptake can occur in the absence of insulin (Wade, Delawder, Reneau and dos Santos, 2020). During exercise there is increased skeletal muscle glucose uptake that can be due to varying factors, such as increased capillarization and rate of glucose uptake (Wade, Delawder, Reneau and dos Santos, 2020). During muscle contraction there is hydrolysis of ATP to ADP and Pi (Wade, Delawder, Reneau and dos Santos, 2020). Increased levels of Ca^{2+} facilitate the cross-bridge between actin and myosin (Wade, Delawder, Reneau and dos Santos, 2020). Due to increased energy demand and decreasing levels of ATP, 5′ AMP-activated protein kinase (AMPK) is activated, which is able to induce GLUT4

translocation to the plasma membrane (Wade, Delawder, Reneau and dos Santos, 2020). Trafficking of GLUT4 to the membrane is believed to occur by TBC1D1 phosphorylation by AMPK (Sakamoto and Holman, 2008). Furthermore, AMPK is associated with enhancement of GLUT1-mediated glucose transport (Abbud et al., 2000).

A summary of the insulin-dependent and non-insulin dependent signaling pathway in the skeletal muscle is presented in Figure 3.

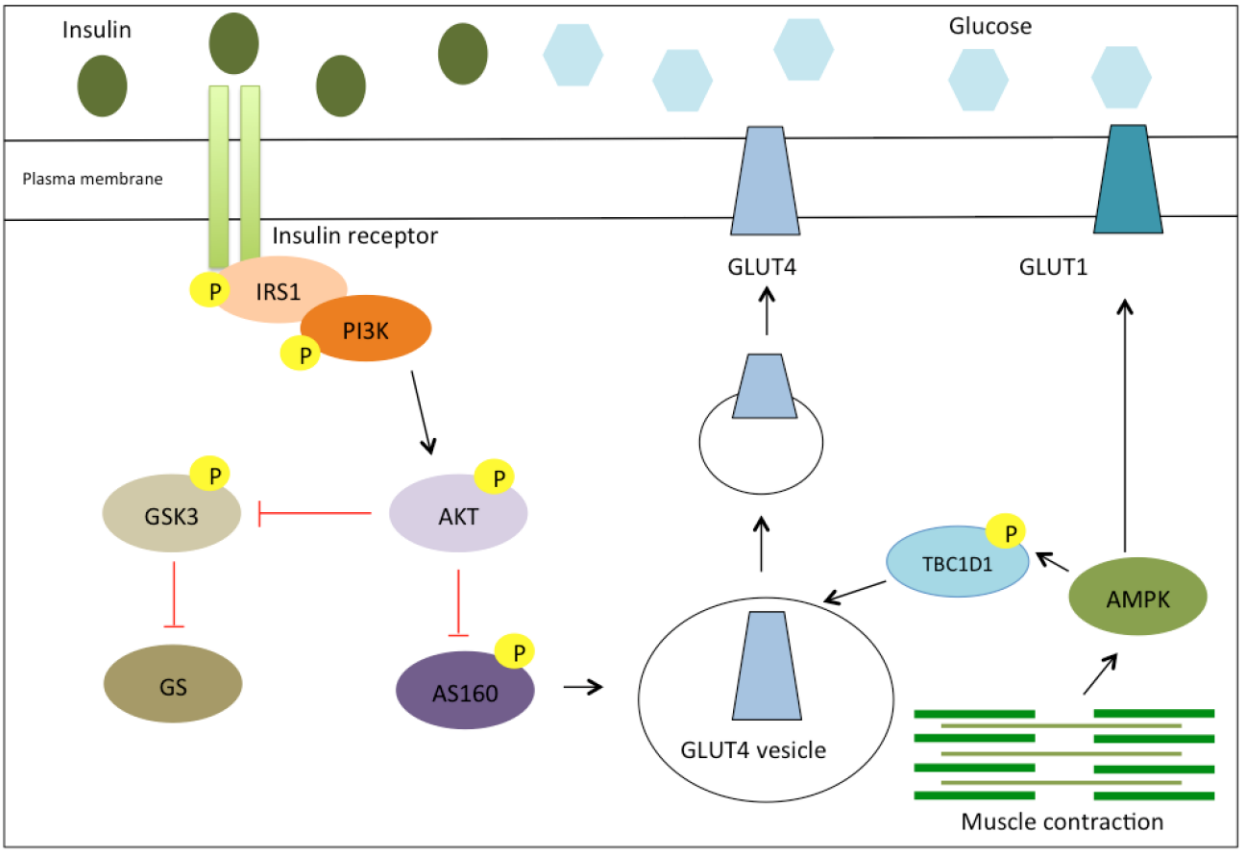

Figure 3. Insulin-dependent and non-insulin dependent signaling pathways in skeletal muscle. Insulin dependent. Insulin binding to the insulin receptor causes tyrosine phosphorylation of the insulin receptor, which is followed by phosphorylation of IRS1. IRS1 then activates PI3K, which then leads to the phosphorylation of Akt. Akt then activates and inhibits AS160, which releases GLUT4 inhibition, and allows for translocation to the membrane. Activated Akt can phosphorylate and inactivate GSK3, which results in active glycogen synthase (GS). Insulin-independent. Activation of AMPK (ie. through muscle contraction) can induce GLUT4 translocation, through phosphorylation of TBC1D1. GLUT1 mediates basal glucose transport into the cell. AMPK can also enhance GLUT1 glucose transport into the cell.

1.3.2 Skeletal muscle glucose homeostasis in the development of type 2 diabetes

Skeletal muscle uptakes approximately 80% of postprandial glucose disposal, therefore is vital for whole-body insulin sensitivity (Sergi et al., 2019). Insulin resistance of the skeletal muscle is an important feature in T2D. Glucose tolerant relatives of individuals with T2D have signs of insulin resistance in the skeletal muscle years before the development of T2D (Vaag, Henriksen and Beck-Nielsen, 1992). Furthermore, patients with T2D show skeletal muscle insulin resistance and impaired muscle glucose uptake, glycogen synthesis, and glycogen synthase activation (Storgaard et al., 2001). Altered glucose homeostasis may be due to defects in the insulin signaling pathway (Storgaard et al., 2001). Specifically, changes in the insulin signaling pathway that disrupt the translocation of GLUT4 to the membrane is linked to glucose intolerance and insulin resistance (Xu et al., 2015). It is suggested that early stages of T2D are linked to reduced IRS and PI3K signaling, whereas late stages of T2D are characterized by reduced GLUT4 translocation (Tremblay, Lavigne, Jacques and Marette, 2001; Kampmann et al., 2011). Rodents with skeletal muscle insulin resistance have been shown to have reduced PI3K activity, resulting in an alteration of the subsequent phosphorylation cascade (Kerouz, Hörsch, Pons and Kahn, 1997). In another study, skeletal muscle of patients with obesity or T2D have reduced IRS1 phosphorylation (Caruso et al., 2014). Hence, it is evident that skeletal muscle insulin resistance involves changes in different aspects of the insulin signaling cascade, which can depend on the stage of T2D.

1.3.3 Mitochondrial Function

Following a meal, there is insulin release and subsequent glucose uptake in peripheral tissues, providing fuel for mitochondrial respiration and ATP production by oxidative phosphorylation. Specifically, a rise in cytoplasmic ATP/ADP in pancreatic β cells signals insulin release (Detimary,

Gilon and Henquin, 1998). The skeletal muscle is rich in mitochondria, and is strongly reliant on oxidative phosphorylation for energy production (Kelley, He, Menshikova and Ritov, 2002). Mitochondrial dysfunction can be defined as reduced mitochondrial content, reduced mitochondrial activity, or changes in reactive oxygen species (ROS) production (Montgomery and Turner, 2015). This can be due to a reduction in mitochondrial biogenesis, mitochondrial content, protein content, and activity of components of the electron transport chain (ETC) (Montgomery and Turner, 2015).

The production of ATP by the mitochondria can be defined by two main steps: the oxidation of NADH or $FADH_2$ and the oxidative phosphorylation of ADP (OXPHOS) (Kim, Wei and Sowers, 2008). NADH and $FADH_2$ can be produced via glycolysis and the Krebs cycle or through β oxidation of fatty acids (Kim, Wei and Sowers, 2008). Briefly, NADH and $FADH_2$ are oxidized while electrons are pumped down the ETC to the final electron acceptor, O_2, ultimately to produce ATP. The electron chain complexes, although able to exist freely, often assemble into a multimeric structure called a supercomplex (Kim, Wei and Sowers, 2008). The most abundant mitochondrial supercomplex contains complexes I, III, and IV (Sergi et al., 2019). It is believed that organizing the complexes into a supercomplex allows for more efficient substrate and electron transfer (Faccioli et al., 2010). In 2015, Antoun et al. showed that there is a reduction in mitochondrial supercomplexes in the skeletal muscle of individuals with T2D, which was associated with reduced mitochondrial respiration (Antoun et al., 2015). The proton gradient generated by the ETC provides energy to produce ATP by ATP synthase (Sergi et al., 2019). Proton leak reduces the proton gradient and occurs due to the presence of uncoupling proteins (UCP) (Kim, Wei and Sowers, 2008). Furthermore, the skeletal muscle isoform of UCP, UCP3, has been shown to reduce the production of ROS (Toime and Brand, 2010). Studies have shown that individuals with T2D

have fewer and smaller mitochondria in skeletal muscle (Morino et al., 2005). Furthermore, the number of mitochondria is directly related to oxidative capacity. Mitochondrial biogenesis is driven by peroxisome proliferator-activated receptor (PPAR) coactivator (PGC)-1, and levels of PGC-1α are higher under conditions of increased ATP demand (ie. exercise) (Kim, Wei and Sowers, 2008). Individuals with insulin resistance and T2D have reduced levels PGC-1α in the skeletal muscle, highlighting a link between mitochondrial number and insulin resistance (Patti et al., 2003).

Mitochondria use fat for energy production, and reduced mitochondrial function and substrate oxidation can lead to ectopic lipid accumulation (Montgomery and Turner, 2015). Two important metabolically active lipids are diacylglycerides (DAG) and ceramides, and ectopic deposition of DAGs and ceramides in organs such as skeletal muscle and liver are strongly associated with insulin resistance (Montgomery and Turner, 2015). Both DAGs and ceramides have been shown to inhibit insulin signaling. Specifically, DAGs have been shown to activate protein kinase C (PKC), which can translocate to the plasma membrane and phosphorylate IRS at a serine residue that lead to inhibition of the insulin signaling pathway (Montgomery and Turner, 2015). Ceramides are negative regulators of insulin activity and have been shown to reduce phosphorylation of Akt, and thus inhibiting the insulin signaling pathway (Montgomery and Turner, 2015). Increased serine phosphorylation of IRS1 and reduced Akt phosphorylation is exhibited in muscle biopsy samples from insulin resistant offspring of patients with T2D (Morino et al., 2005). Thus, lipid accumulation due to mitochondrial dysfunction highlights a plausible link to insulin resistance (Montgomery and Turner, 2015).

Changes in mitochondrial dynamics have also been linked to the development of insulin resistance (Kelley, He, Menshikova and Ritov, 2002). As a quality control mechanism, the mitochondria is

constantly undergoing fusion and fission, which is critical for maintaining mitochondrial DNA, respiratory capacity, and cellular response to stress (Lin et al., 2018). There have been reports that individuals with insulin resistance or T2D have reduced levels of mitofusin 2 (Mfn2) and decreased fusion (Lin et al., 2018). Fusion is important for the exchange of material between healthy mitochondria, therefore, lack of fusion leads to a reduced number of healthy mitochondria (Lin et al., 2018). An increase in mitochondrial fission in the muscle leads to insulin resistance, and improved fusion can improve insulin sensitivity (Jheng et al., 2011). Therefore, mitochondrial dynamics presents a link between reduced mitochondrial number and increased mitochondrial damage and insulin resistance.

Several studies have shown that the skeletal muscle in individuals with T2D or those with a family history of T2D have reduced mitochondrial function (Montgomery and Turner, 2015). In healthy individuals, hyperinsulinemia leads to increased ATP production, however, individuals with insulin resistance often have a blunted response to insulin (Asmann, Stump and Short, 2006). For example, in one study there was reduced ATP production following insulin infusion in patients with T2D compared to healthy controls (Asmann, Stump and Short, 2006). In another study, Ritov et al., (2005) showed that there is reduced mitochondrial number and electron transport activity in T2D and obese patients compared to lean patients. These results are consistent with a study investigating the effects of IRS1 deletion in mice (Long, Cheng, Copps and White, 2010). These knockout mice have diminished insulin sensitivity, impaired oxidative phosphorylation, and reduced ATP production (Long, Cheng, Copps and White, 2010). Therefore, mitochondrial dysfunction may not only be a cause of insulin resistance, but also a consequence. Taken together, it is apparent that mitochondrial impairments play an important role in the development of skeletal muscle insulin resistance and T2D.

1.3.4 Reactive oxygen species

Oxidative stress can be defined as excess oxidative species that can damage cells and alter signaling pathways. Reactive species include ROS such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ions (OH^-) (Hurrle and Hsu, 2017). The production of ROS occurs normally at low physiological levels, and major sites include mitochondrial ETC, endoplasmic reticulum, and the peroxisome (Hurrle and Hsu, 2017). Under normal conditions, about 0.2-2% of electrons leak in the ETC and interact with oxygen to create oxygen species (Turrens, 2003). The generation of ROS has important roles in living organisms. Processes such as protein phosphorylation, activation of transcription factors, apoptosis, and differentiation are dependent on physiological ROS production. Normal levels of H_2O_2 and O_2^- are important for signal transduction in pancreatic cells and are hypothesized to regulate insulin secretion (Newsholme, Cruzat, Arfuso and Keane, 2014). Complexes I and III are the main sites of ROS production in the mitochondria (Zhao, Jiang, Zhang and Yu, 2019). H_2O_2 production is the byproduct of superoxide degradation, by superoxide dismutase (SOD) enzymes such as SOD2 (Anderson et al., 2009). An imbalance between ROS and the ability of the biological system to detoxify reactive species can be detrimental and lead to oxidative stress (Newsholme et al., 2016).

Decreased substrate oxidation due to defects in OXPHOS is related to increased electron leakage in the electron transport chain and the formation of superoxides (Montgomery and Turner, 2015). Increased ROS can lead to mitochondrial DNA damage, protein aggregation, and lipid peroxidation, which can eventually lead to mitophagy (Montgomery and Turner, 2015). Removal of mitochondria, for example through mitophagy, can generate feedback loop since reduced mitochondria number and function can further reduce substrate oxidation and lipid accumulation (Montgomery and Turner, 2015).

Cells have antioxidants such as glutathione peroxidase, and enzymes such as glutaredoxin, catalase, and SOD to counteract oxidative stress. Glutathione peroxidases (GPx) function to reduce lipid hydroperoxides to alcohols and H_2O_2 to water. Glutaredoxins (Grx) are enzymes that function as cofactors of glutathione peroxidase. Specifically, glutaredoxins are oxidized by substrates, and regenerated through reduction by glutathiones. Increased metabolic activity ofthe skeletal muscle (ie. through exercise) leads to increased production of oxidants (Powers, Ji and Leeuwenburgh, 1999). It has been shown that there are increased levels of GPx following exercise, which likely functions to reduce elevated ROS (Powers, Ji and Leeuwenburgh, 1999). Catalase is an enzyme that catalyzes the decomposition of H_2O_2 to water and oxygen (Lee et al., 2010). In one study, targeted overexpression of catalase in the mitochondria of muscle in mice prevented lipid-induced insulin resistance (Lee et al., 2010). As mentioned earlier, SOD is an enzyme that catalyzes the conversion of O_2^- into O_2 and H_2O_2 (Kang et al., 2014). Heterozygous knockout mice for SOD2 that were fed a high fat diet, were shown to have accelerated oxidative stress and impaired insulin sensitivity in the skeletal muscle (Kang et al., 2014).

Recent studies have indicated that ROS production is linked to many chronic diseases including T2D. In one study, rodents fed a high-fat diet had increased H_2O_2 production which corresponded to insulin resistance of the skeletal muscle (Anderson et al., 2009). Interestingly, increased ROS and insulin resistance was prevented by giving rodents antioxidants or over expressing catalase (Anderson et al., 2009). In another study, the involvement of oxidative stress in insulin resistant myocytes was confirmed. L6 myocytes that were exposed to H_2O_2 to cause oxidative stress, had reduced insulin-stimulated glucose uptake (Maddux et al., 2001). Interestingly, the addition of alpha-lipoic acid, an antioxidant, protected insulin action and improved glucose uptake (Maddux et al., 2001). Therefore, it is clear that although physiological levels of ROS are important for

normal cell functioning, increased ROS production can have adverse effects such as the development of insulin resistance in skeletal muscle.

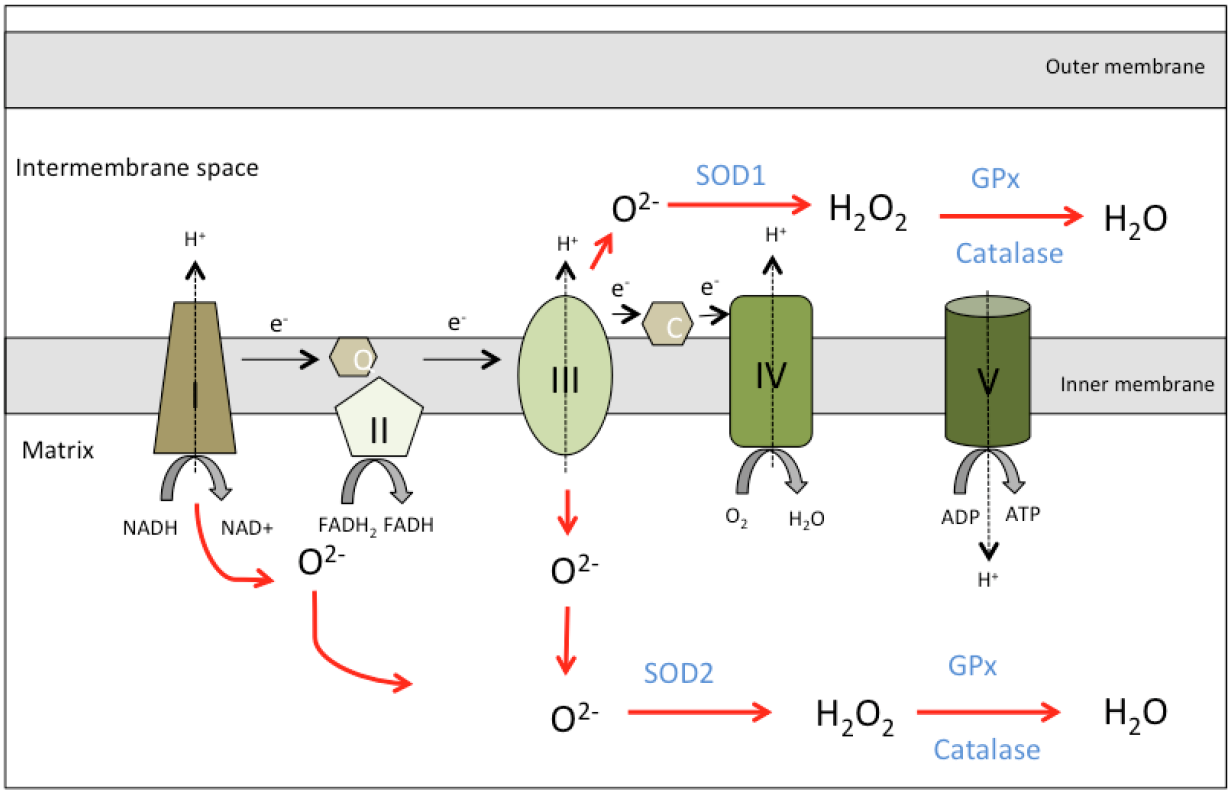

Figure 4. Schematic representation of mitochondrial ROS production. Electrons (e^-) from NADH and $FADH_2$ go down the electron transport chain and reduce O_2 to form H_2O at complex IV. Complex I and III are the main sites for ROS in the cell, due to leakage of e^- from the electron transport chain which can lead to the production of O_2^-. Superoxide degradation can occur by SOD1 in the intermembrane space or SOD2 in the mitochondrial matrix. Glutathione peroxidase function to reduce H_2O_2 to water. Catalase functions similarly to GPx and reduces H_2O_2 to water. Figure adapted from Li et al., 2013.

1.4 Adipose tissue perturbations in type 2 diabetes

The obesity epidemic is closely linked to the T2D epidemic. Obesity is considered a serious health concern since it is associated with several health conditions such as T2D, cardiovascular disease, hypertension, and hyperlipidemia (Blüher, 2016). Furthermore, it is considered one of the greatest risk factors for the development of insulin resistance (Blüher, 2016). Adipose tissue plays an important role in whole-body homeostasis, as it functions not only as a storage reservoir, but also acts as an endocrine organ (Friedman et al., 1991). As an endocrine organ, adipose tissue secretes adipokines, cytokines, and chemokines, which signal to organs such as the skeletal muscle and brain to regulate metabolism (Blüher, 2016). There are two types of adipose tissue, white adipose tissue (WAT), which is used for energy storage and the secretion of adipokines, and brown adipose tissue (BAT), which is primarily used for thermogenesis (Blüher, 2016). Adipose tissue consists of different cell types, including adipocytes, fibroblasts, endothelial cells, and immune cells (Bourlier et al., 2008). Triglycerides (TGs) are stored in adipocytes in adipose tissue, which can be hydrolyzed by the process of lipolysis into fatty acids (FA) and glycerol and released into the circulation for uptake into peripheral tissue such as the skeletal muscle (Blüher, 2016). Lipolysis is normally inhibited by insulin signaling in adipocytes. During fasting, there is an increase in free fatty acids (FFA) in the circulation, to provide energy for peripheral tissues. Elevated systemic FFA is believed to contribute to obesity associated insulin resistance (Manna and Jain, 2015). Specifically, increased FFA is associated with increased inflammation of the adipose tissue, which can cause metabolic disturbances in peripheral tissues (Manna and Jain, 2015). In addition, ectopic lipid accumulation, in organs such as the muscle and liver, is linked to metabolic dysfunction, and is a strong predictor of insulin resistance in both lean individuals and individuals with obesity.

Adipose tissue is considered to have a high degree of plasticity, especially in response to changing demand for fat storage. When there is increased demand for fat storage, adipocytes may undergo hypertrophy (increasing size) or hyperplasia (increasing number) (Blüher, 2016). Hypertrophic adipose tissue morphology is linked with insulin resistance, whereas a hyperplastic morphology is linked with improved metabolic parameters (Haczeyni, Bell-Anderson and Farrell, 2017). Excess nutrient uptake, and subsequent adipocyte hypertrophy, can lead to cytokine secretion from the adipose tissue, which can also lead to increased hyperplasia (Haczeyni, Bell-Anderson and Farrell, 2017). This occurs to maintain normal levels of lipids in the circulation. However, sustained excess nutrient uptake leads to adipose tissue stress, inflammation, and altered adipokine release, which can then affect the functioning of other tissues (Haczeyni, Bell-Anderson and Farrell, 2017).

1.4.1 Inflammation and adipokine release

Low grade chronic inflammation of adipose tissue is linked to metabolic disturbances and insulin resistance development. Adipokines are cytokines released from adipose tissue and can have pro-inflammatory or anti-inflammatory effects (Makki, Froguel and Wolowczuk, 2013). Collectively, adipokines can exert their effects in an autocrine, paracrine or systemic manner, and can alter glucose metabolism and immunity (Makki, Froguel and Wolowczuk, 2013). Anti-inflammatory adipokines include adiponectin, transforming growth factor β (TGFβ), interleukin-10 (IL-10), IL-1, and IL-4, whereas pro-inflammatory adipokines include IL-6, IL-1β, and leptin (Makki, Froguel and Wolowczuk, 2013). It is well documented that changes in adiponectin, IL-6, TNF-α, and leptin, are implicated in the development of insulin resistance (Makki, Froguel and Wolowczuk, 2013). Although adipose tissue inflammation is strongly related to obesity, not all individuals who develop adipose tissue inflammation are obese (Burhans et al., 2018). It is possible that other factors such as chemicals can induce cell stress, which can in turn lead to adipose tissue

inflammation. Furthermore, adipokines secreted from adipose tissue have been associated with skeletal muscle insulin resistance (Havekes and Sauerwein, 2010). In this chapter, we will focus on some adipokines implicated in adipose tissue and skeletal muscle insulin resistance and the development of T2D.

1.4.1.1 Adiponectin

Adipocytes secrete adiponectin, an adipokine that is inversely related to adiposity (Nigro et al., 2014). Studies have shown that plasma adiponectin levels are directly correlated with insulin sensitivity. In one study, mice without WAT had almost no circulating adiponectin, and were insulin resistant (Yamauchi et al., 2001). However, when adiponectin was administered to these mice, insulin sensitivity was improved, and there was a reduction in muscle and hepatic TG accumulation (Yamauchi et al., 2001). In addition, it has been shown that treatment with thiazolidinedione (TZD), an anti-diabetic drug that promotes the uptake of FA into adipose tissue, leads to increased adiponectin levels in both mice and humans (Maeda et al., 2001). Although adiponectin is inversely related to adiposity, and TZDs promote lipid accumulation, the insulin sensitizing effects of TZDs may be due to acceleration of the production of adiponectin (Kubota et al., 2006). Adiponectin exerts its actions through binding the adiponectin receptors (AdipoR) AdipoR1 and AdipoR2 (Almabouada et al., 2012). Binding to these receptors leads to the activation of signaling pathways such as AMPK and PPARα (Almabouada et al., 2012). This can lead to increased FA oxidation and glucose uptake in the skeletal muscle. In vitro studies have shown that adiponectin increases insulin sensitivity in the skeletal muscle (Yamauchi et al., 2002). C2C12 muscle cells incubated with adiponectin for 60 minutes show increased glucose uptake and β-oxidation (Yamauchi et al., 2002). Moreover, this group showed that adiponectin was able to increase acetyl-CoA carboxylase (ACC) and AMPK phosphorylation in myocytes, and the

activation of AMPK is shown to stimulate glucose uptake (Yamauchi et al., 2002). Similarly, L6 muscle cells exposed to adiponectin for 4 hours were shown to have increased GLUT4 translocation and glucose uptake, which is consistent with increased activation of AMPK (Ceddia et al., 2004). Therefore, it is suggested that impaired adiponectin function contributes to insulin resistance in organs such as the skeletal muscle, which can contribute to the development of T2D.

1.4.1.2 Interleukin-6

Interleukins are a group of cytokines that can be secreted from adipose tissue and are often increased in obesity and T2D. IL-6 is an interleukin that can act both as a pro-inflammatory and anti-inflammatory cytokine, and is secreted by different cells in adipose tissue such as adipocytes and macrophages (Kern et al., 2018). IL-6 protein content has been shown to be negatively associated with insulin-stimulated glucose disposal in adipose tissue, and chronic IL-6 has been associated with impaired glucose metabolism (Nieto-Vazquez, Fernandez-Veledo, de Alvaro and Lorenzo, 2008). When secreted from the adipose tissue, IL-6 acts as a pro-inflammatory cytokine and is directly correlated with adiposity, fat mass, and insulin resistance (Makki, Froguel and Wolowczuk, 2013). Specifically, IL-6 is associated with insulin resistance in adipose tissue since it upregulates suppressor of cytokine signaling 3 (SOCS3) which impairs IRS1 phosphorylation (Nieto-Vazquez, Fernandez-Veledo, de Alvaro and Lorenzo, 2008). Furthermore, IL-6 has been shown to increase lipolysis in adipocytes, increasing the levels of FFA, and fat oxidation. Interestingly, after exercise the skeletal muscle has also been shown to also secrete IL-6, however, it appears to have anti-inflammatory effects (Nieto-Vazquez, Fernandez-Veledo, de Alvaro and Lorenzo, 2008). Reports have shown that IL-6 secreted from the skeletal muscle is associated with improved substrate metabolism and whole-body glucose metabolism (Glund et al., 2007). This discrepancy in IL-6 function may be due to differences in exposure time. When skeletal muscle

cells are exposed to IL-6 acutely, there is an additive effect with insulin, leading to increased activity of the insulin signaling pathway and glucose uptake (Nieto-Vazquez, Fernandez-Veledo, de Alvaro and Lorenzo, 2008). However, chronic exposure, such as when IL-6 is being released from the adipose tissue in obesity, leads to impaired GLUT4 translocation in the skeletal muscle (Nieto-Vazquez, Fernandez-Veledo, de Alvaro and Lorenzo, 2008). Another possible explanation for this discrepancy is the differences in IL-6 levels released from tissues (Langberg, Olesen, Gemmer and Kjaer, 2002). It has been shown that there are higher levels of IL-6 from peritendinous tissue compared to the contracting skeletal muscle (Langberg, Olesen, Gemmer and Kjaer, 2002). Therefore, IL-6 appears to have a dual effect, either in the adipose tissue or the skeletal muscle, and the effects may be influenced by whether elevated levels of IL-6 is acute or chronic, which may also be influenced by the concentration of IL-6 released.

1.4.1.3 Fatty acid binding protein 4

Fatty acid binding protein 4 (FABP4), which is sometimes referred to as adipocyte P2 (aP2) is a novel proinflammatory adipokine, that makes up approximately 1% of all soluble proteins in adipose tissue (Trojnar et al., 2019). It was first described in 2006 as a circulating protein, and shortly after, several studies have investigated its effects in humans (Kralisch and Fasshauer, 2012). It is an intracellular FA carrier that functions by binding to hydrophobic ligands and trafficking FA in cells (Trojnar et al., 2019). It is also released from adipocytes and macrophages and is positively related to obesity and insulin resistance (Nakamura et al., 2017). This FA transporter also plays an important role in FA uptake into the muscle (Syamsunarno et al., 2013). Mice that have a whole body knockout for FABP4 have defected FA uptake into muscle, with compensatory increased glucose consumption for energy (Syamsunarno et al., 2013). FABP4 is normally secreted under obesogenic conditions (Trojnar et al., 2019). Obese mice that have a

FABP4 deletion from preadipocytes decreased insulin levels and increased adiponectin concentration, suggesting improved insulin sensitivity (Furuhashi et al., 2007). Increased FABP4 secretion has been reported to enhance hepatic gluconeogenesis, and reduce glycolysis in the muscle and liver (Trojnar et al., 2019). It is also suggested that insulin resistance may impair insulin suppression of FABP4 release (Trojnar et al., 2019). This in turn can feedback and lead to enhanced insulin release to compensate, which can exacerbate insulin resistance. FABP4 also inhibits proteins in the Akt pathway (Trojnar et al., 2019). Furthermore, FABP4 has been shown to trigger the ubiquitination and degradation of PPARγ, which is important in adipogenesis and insulin sensitivity (Trojnar et al., 2019). Interestingly, FABP4 null mice have increased PPARγ levels and enhanced adipogenesis (Garin-Shkolnik, Rudich, Hotamisligil and Rubinstein, 2013). In addition, higher levels of FABP4 and lower levels of PPARγ have been reported in visceral adipose tissue compared to subcutaneous adipose tissue (Trojnar et al., 2019). This is interesting since visceral tissue is associated with metabolic dysfunctions connected to cardiovascular disease and diabetes; therefore, FABP4 levels in these tissues may explain some of the morphological and functional differences between these adipose tissue depots (Trojnar et al., 2019). Together, it is evident that changes in FABP4 secretion from the adipose tissue may play a role in the development of insulin resistance and T2D.

1.4.1.4 Tumor necrosis factor

Tumor necrosis factor-α (TNF-α) is a proinflammatory cytokine secreted by adipocytes and adipose-tissue resident macrophages (Plomgaard et al., 2007). Type 2 diabetes is associated with elevated levels of TNF-α in skeletal muscle, adipose tissue, and plasma (Plomgaard et al., 2007). Increased levels of TNF-α is closely related to insulin resistance, as shown in both animal and human studies. For example, TNF-α causes insulin resistance through inhibiting the insulin

signaling pathway in muscle and adipose tissue from obese mice (Hotamisligil et al., 1996). Specifically, TNF-α has been shown to cause increased phosphorylation of IRS1 at Ser 307 in murine adipocytes (or Ser 312 in humans), which makes it an inhibitor of insulin receptor tyrosine kinase activity, and thus inhibiting the downstream insulin signaling cascade (Hotamisligil et al., 1996). In the skeletal muscle, one hour treatment with TNF-α before treatment with insulin was shown to result in increased phosphorylation of p70 S6 kinase (S6K) and extracellular signal-regulated kinases (ERK)-1/2, and c-Jun N-terminal kinase (JNK) in skeletal muscle and increased phosphorylation of IRS1 at Ser 312, all which is implicated in the negative regulation of insulin signaling (Plomgaard et al., 2005). *In vivo* studies have shown that TNF-α levels are elevated in obese, insulin resistant mice, whereas obese mice with a TNF-α deletion are protected against insulin resistance. In healthy humans, infusion of TNF-α inhibits peripheral glucose uptake, without preventing endogenous gluconeogenesis (Plomgaard et al., 2005). Correspondingly, humans with metabolic disease with long term treatment with an anti-TNF-α were shown to improve fasting blood glucose and to increase adiponectin levels (Stanley et al., 2011). TNF-α and IL-6 are early mediators of inflammation, and work closely together (Plomgaard et al., 2005). TNF-α induces the production of IL-6, which in turn can contribute to skeletal muscle insulin resistance, since chronic IL-6 has been reported to reduce insulin-stimulated glucose uptake in myocytes (Plomgaard et al., 2005). TNF-α is also associated with insulin resistance in adipose tissue. Specifically, TNF-α has been shown to inhibit insulin stimulated autophosphorylation of the insulin receptor and tyrosine phosphorylation of IRS1 (Hotamisligil et al., 1996). Therefore, increased TNF-α levels are linked to reduced insulin signaling, and insulin resistance.

1.4.1.5 Leptin

Leptin is an adipokine that is secreted by WAT, and levels are positively correlated with adiposity (Kelesidis, Kelesidis, Chou and Mantzoros, 2010). Leptin functions as a satiety hormone and mediates its effects by binding to leptin receptors in the brain and in peripheral tissues (Kalra, 2008). Circulating leptin plays an important role in energy homeostasis, as it functions as an indicator for energy reserve levels and directs the central nervous system to adjust food intake and energy expenditure (Kelesidis, Kelesidis, Chou and Mantzoros, 2010). Patients with leptin deficiency due to gene mutations or leptin resistance are often obese due to hyperphagia (Strobel et al., 1998). Leptin resistance is defined as increased circulating levels of leptin but an absence in leptin mediated response (Kalra, 2008). Furthermore, obese and diabetic mice that have leptin receptor mutations have insulin resistance, in addition to other features of metabolic syndrome (Kelesidis, Kelesidis, Chou and Mantzoros, 2010). In a 2014 study, Guadalupe-Grau et al. showed that leptin can increase activity of IRS-1 and subsequent GLUT4 translocation in the skeletal muscle. In skeletal muscle, leptin has been shown to stimulate phosphorylation of AMPK and increase FA oxidation (Minokoshi et al., 2002). Together, it is evident that leptin plays an important role in whole-body energy homeostasis, and reduced leptin or leptin deficiency has important implications in the development of metabolic disease.

1.4.1.6 Other adipokines

There are several other adipokines that have been linked to insulin resistance and the development of T2D. Their functions and implications in T2D have been summarized in Table 3.

Table 3. Metabolic function and dysfunction of adipokines implicated in the development of insulin resistance and T2D.

Adipokine	Metabolic Function	Metabolic Dysfunction	Reference
Monocyte chemoattractant protein (MCP-1)	Recruits monocytes and macrophages	↑ in adipose tissue of obese mice ↓ insulin stimulated glucose uptake in adipocytes	(Sartipy and Loskutoff, 2003)
Chemerin	Anti-inflammatory ↑ insulin-stimulated glucose uptake and IRS-1 phosphorylation (3T3-L1 adipocytes)	Expression of human chemerin in mice leads to skeletal muscle insulin resistance	(Becker et al., 2010)
Resistin	↑ TNF-α and IL-6 expression	↑ serum levels in individuals with T2D ↑ insulin resistance in mice	(Steppan et al., 2001)
Vaspin	Insulin-sensitizing Anti-inflammatory	Secreted from visceral adipose tissue ↓ with worsening diabetes	(Liu et al., 2018)
Omentin	Insulin-sensitizing	↓ plasma levels in obesity	(de Souza Batista et al., 2007)
Plasminogen activator inhibitor (PAI-1)	Negative regulator of fibrinolysis	↑ circulating levels in obesity and insulin resistance PAI-$1^{-/-}$ mice on high fat diet have improved insulin sensitivity	(Ma et al., 2004)

1.5 Bisphenols and metabolic disturbances

Several studies have investigated the effects of selective ER modulators (SERMs), which bisphenols are sometimes considered, on glucose metabolism and inflammation (Shen et al., 2019). The estrogen agonist raloxifene, a drug that is primarily used in postmenopausal women, has been shown to improve peripheral insulin sensitivity, as it increases adiponectin levels while reducing levels of proinflammatory cytokines, and other adipogenic markers such as FABP4 (Shen et al., 2019). Whereas, another SERM, tamoxifen, which is used in the treatment of breast cancer, has been linked to increased risk for the development of T2D (Xu, Lovre and Mauvais-Jarvis, 2017). This may be due to tamoxifen binding to the ER of pancreatic β cells and impairing pancreatic islet survival (Xu, Lovre and Mauvais-Jarvis, 2017). Therefore, it is clear that the binding of different SERM's to the ER can lead to different physiological outcomes. Similarly, bisphenols have been shown to bind to the ER of different cell types such as adipocytes and muscle cells, and cause changes in glucose metabolism, however, certain pathways following bisphenol exposure have not been completely characterized.

1.5.1 Bisphenols and metabolic disturbances in muscle

The skeletal muscle accounts for a majority of glucose disposal within the body, therefore, is crucial in regulating whole-body glucose metabolism (DeFronzo and Tripathy, 2009). Although certain EDCs such as bisphenols have been shown to be associated with insulin resistance in the skeletal muscle, it is not completely clear how this occurs. There are seldom studies investigating the effects of BPA on skeletal muscle insulin resistance, even though the muscle accounts for more than 80% of postprandial glucose disposal (DeFronzo and Tripathy, 2009). In one study, rats were treated with 10-400 mg/kg bw/day of BPA through oral incubation for 30 days (Mullainadhan,

Viswanathan and Karundevi, 2017). Insulin signaling was measured in the gastrocnemius muscle of these rats and it was shown that insulin receptor β protein and its tyrosine phosphorylated form were reduced at both low and high doses of BPA (Mullainadhan, Viswanathan and Karundevi, 2017). Total IRS1 and phosphorylated IRS1, which is important for activation of insulin signaling pathways, were reduced (Mullainadhan, Viswanathan and Karundevi, 2017). Not surprisingly, total Akt and phosphorylated Akt were reduced as well, in a non-monotonic manner (Mullainadhan, Viswanathan and Karundevi, 2017). These changes in the insulin signaling pathway of the muscle corresponded to hyperglycemia and insulin resistance in these rats (Mullainadhan, Viswanathan and Karundevi, 2017). In addition, they also showed that BPA was able to accumulate in the gastrocnemius muscle of these rats, following a non-monotonic dose response. This may be due to the fact that at higher concentrations, BPA starts becoming toxic to the skeletal muscle, therefore, accelerating the clearance faster at higher doses than at lower doses (Mullainadhan, Viswanathan and Karundevi, 2017). In another study, mice given 50 μg/kg bw/day of BPA orally for 12 weeks had reduced phosphorylation of Akt and GSK3 in the skeletal muscle, which correspond to glucose intolerance in these mice (Moon et al., 2015). Interestingly, these mice had reduced levels of adiponectin, which is known to improve insulin sensitivity in the skeletal muscle, therefore, highlighting the communication between the adipose tissue and the skeletal muscle (Moon et al., 2015). Contrastingly, Alonso-Magedelena et al. (2006) showed that a single low dose of BPA (10 μg/kg bw/day) administered subcutaneously has been shown to rapidly increase plasma insulin and decrease blood glucose 30, 60, and 90 minutes after injection. This group then decided to measure the effects of longer, or "chronic" BPA exposure, by subcutaneously administering 10 or 100 μg/kg bw/day of BPA to mice for 4 days (Alonso-Magdalena et al., 2006). They found increased insulin release at both doses, however, mice given

100 μg/kg bw/day had more potent hyperinsulinemia (Alonso-Magdalena et al., 2006). Furthermore, they highlighted that a concentration of 100 μg/kg/day is comparable to that found in the late pregnancy. Therefore, it is evident that environmentally-relevant concentrations of BPA are able to alter muscle insulin signaling and glucose metabolism *in vivo*. It is important to note, however, that the effects of BPA on glucose metabolism seem to depend on the length of treatment. Acute BPA treatments appear to improve aspects of glucose metabolism, such as glucose clearance, however, chronic treatments appear to result in glucose intolerance. Furthermore, since these experiments have been performed *in vivo*, it is unclear whether these results are due to direct interaction of BPA with skeletal muscle cells, or indirectly through altered metabolism in other tissues.

Taken together, it is evident that BPA exposure has an effect on skeletal muscle metabolism *in vivo*. However, since many studies have been done *in vivo*, it is currently unclear whether the effects of BPA and BPS are due to the effects of direct exposure or through communication. Therefore, more research is required to determine the effects of direct BPA and BPS on skeletal muscle metabolism.

1.5.2 Bisphenols and metabolic disturbances in adipocytes and adipose tissue

Epidemiological studies have shown a positive correlation between BPA exposure and obesity, however, *in vitro* and *in vivo* studies often have conflicting results regarding the effects of BPA on adipocyte metabolism (Ohlstein et al., 2014). Previous studies have shown that BPA enhances adipogenesis through ER-mediated pathways in a concentration dependent manner in human adipose derived stromal cells (ASCs) (Ohlstein et al., 2014). In 2015, Boucher et al. demonstrated that BPA-G is not an inactive metabolite, as previously believed, and instead can alter adipocyte metabolism. They showed that chronic exposure to 0.05 and 0.25 μM of BPA-G induces

differentiation in 3T3-L1 adipocytes and primary human adipocytes. Furthermore, 10 μM of BPA-G increased mRNA expression of adipogenic factors such as sterol regulatory element-binding protein 1 (SREBF1) and lipoprotein lipase (LPL), which play important roles in mature adipocyte phenotype (Atlas, Boucher, Ahmed and Boudreau, 2015). Moreover, it was previously believed that BPA-G did not bind ER-α and -β, however, Boucher et al. (2015) found that using an ER antagonist prevented BPA-G (1 and 10 μM) induced adipogenesis in 3T3-L1 preadipocytes.

Interestingly, other studies have found that BPA fails to induce adipogenesis at similar concentrations used in previous studies. For example, De Filippis et al. (2018) used the same concentrations of BPA that have been shown to enhance adipogenesis in adipocytes (1-100 nM) and found no effect on adipogenesis in 3T3-L1 adipocytes (De Filippis, Li and Rosen, 2018). This inconsistency was also encapsulated in another study, where BPA failed to induce adipogenesis in mesenchymal stem cells (MSCs), but instead only in 3T3-L1 cells (Chamorro-García et al., 2012). These differences may be due to differences in mechanisms of BPA and BPA-G, differences in cell types (3T3-L1, primary human adipocytes etc.), or the use of more refined techniques. Furthermore, in the study by Boucher et al. (2015), the differentiation cocktail used included either BPA-G or dexamethasone, in which dexamethasone is normally required for differentiation. Interestingly, in the study by De Filippis et al. (2018) and Chamorro-García et al. (2012) they use both BPA and dexamethasone in the differentiation cocktail. Therefore, it is also possible that by using both BPA and dexamethasone it is not possible to further increase differentiation when using both BPA and dexamethasone.

Adipose tissue from individuals with T2D have reduced insulin receptor kinase activity, which corresponds to reduced insulin stimulated lipogenesis, and glucose transport into the adipose tissue (Lonnroth, Digirolamo, Krotkiewski and Smith, 1983). Adipocytes derived from obese or T2D

patients have reduced IRS1 phorsphorylation, which appears to be one of the first changes in the insulin signaling pathway (Danielsson, Öst, Nystrom and Strålfors, 2005). In one study, 3T3-L1 preadipocytes treated chronically with 1 nM of BPA during differentiation have reduced insulin-stimulated phosphorylation of Akt and glucose uptake (De Filippis, Li and Rosen, 2018). Furthermore, they showed that IL-6 and TNF-α expression was increased in 3T3-L1 adipocytes treated with 1 or 3 nM of BPA (De Filippis, Li and Rosen, 2018). Interestingly, in a different study, 3T3-F442A adipocytes treated with 10^{-4} M of BPA for 24 hours had increased glucose uptake and GLUT4 protein levels, therefore emphasizing the difference effects that can occur depending on the concentration of BPA and length of exposure (Sakurai et al., 2004).

Fetal exposure to BPA at levels at or lower than the established daily human safe dose (50 µg BPA/kg bw/day) has been shown to increase body weight and postnatal growth in *in vivo* studies. In one study, pregnant rodents administered 10 mg/L of BPA orally, had 10–25 ng/g of BPA in tissue, which is comparable to human samples (Desai et al., 2018). They showed that maternal BPA exposure induced offspring obesity, hypertrophy of adipocytes, and increased expression of adipogenic and lipogenic factors (Desai et al., 2018). This was confirmed in *in vitro* studies where pre-adipocytes from rat incubated with 1-20 µM of BPA for 5 days had increased number of adipocytes, increased expression of adipogenic transcription factors (PPARγ, C/EBPα), and increased expression of TNFα (Desai et al., 2018). In a similar study, rats exposed to 0.5 µg/kg bw/day of BPA orally from gestational day 3.5 until postnatal day 22, which is 8-10 times lower than European Food and Safety Authority daily tolerable dose, had higher plasma triglyceride concentrations and inguinal WAT adipocyte density in rat offspring (Lejonklou et al., 2017). It appeared that there was adipocyte hyperplasia, which is suggested to occur in the early stages of development. In addition, hyperplasia may make the offspring more at risk for being

overweight later in life due to the ability to store more fat (Lejonklou et al., 2017). Therefore, gestational exposure to BPA can lead to lifelong adverse effects on adipose tissue metabolism in offspring.

Although the effects of BPS on adipocytes are even less understood, BPS has been shown to increase lipid accumulation and gene expression of adipogenic markers in primary human adipocytes (Boucher, Ahmed and Atlas, 2016). In one study, treatment of 3T3-L1 cells with high concentrations of BPS (10 μM) during differentiation induced lipid accumulation and increased expression of adipogenic markers (Ahmed and Atlas, 2016). Furthermore, they showed that BPA and BPS can activate PPARγ, which is required for BPA- and BPS-induced adipogenesis (Ahmed and Atlas, 2016). Interestingly, BPS binding to the nuclear receptor PPARγ is more enhanced than BPA, thus implicating that BPS may not be a safe alternative for BPA (Ahmed and Atlas, 2016).

Low doses of BPA have been shown to alter adipokine expression in adipocytes. Cimmino et al. (2019) showed that human mature adipocytes and stromal vascular cells (SVFs) from subcutaneous mammary adipose tissue treated with 0.1 nM of BPA for 24 or 48 hours had increased levels of IL-6 and MCP1α via GPR30 (Cimmino et al., 2019). In another study, mice fed BPA (5, 50 (TDI), 500, or 5000 μg/kg/day) for 30 days had increased body weight and fat mass in a nonmonotonic manner (Yang et al., 2016). This corresponded to increased circulating inflammatory markers, such as leptin and TNF-α and local inflammation in WAT (Yang et al., 2016). In addition, human pre-adipocytes isolated from human adipose tissue explants incubated for 6 hours with 0.1, 1, and 10 nM of BPA have been shown to have reduced levels of adiponectin (Hugo et al., 2008). This is important since adiponectin is a critical adipokine involved in insulin sensitivity and inflammation and has been shown to be reduced in T2D (Hugo et al., 2008). This is consistent with a study by Menale et al. (2016) that found a strong inverse association between

BPA and adiponectin in children with obesity (Menale et al., 2016). Furthermore, they found that resistin, an adipokine which increases TNF-α and IL-6 expression, was detected in adipocytes only after BPA treatment (Menale et al., 2016). Taken together, it is evident that BPA can alter inflammation and adipokine levels in adipose tissue, which may be able to contribute to metabolic dysfunctions such as the development of insulin resistance.

Together, these studies have suggested that BPA may not only be an obesogenic compound but also diabetogenic. Understanding the effects of bisphenols on adipocytes and adipose tissue provides insight to its effect on whole-body metabolism, however, discrepancies in results may be due to differences in cell types used in the above discussed studies. Since whole adipose tissue contains preadipocytes, fibroblasts, endothelial cells, and immune cells such as macrophages, investigating the effects of bisphenols on whole adipose tissue may provide insight on the effects that occur *in vivo*.

1.6 Significance

It is clear that humans are continuously exposed to bisphenols, as they have been found at metabolically active concentrations in urine samples of nearly everyone tested. The effects of bisphenols on skeletal muscle metabolism and adipose tissue have not been completely elucidated. Understanding the effects of bisphenols on both skeletal muscle and adipose tissue metabolism will provide insight into the mechanism(s) by which bisphenols contribute to insulin resistance and T2D. Furthermore, using bisphenols at concentrations that cover both physiological and supraphysiological concentrations is important in modeling the non-monotonic dose response feature of bisphenols. The mass production of bisphenols, chronic exposure, and its reported effects on endocrine disruption calls for the need to continue research on its effects on human

health. This will provide thorough information to public health agencies on the effects of bisphenols on health in order to improve manufacturing regulations.

2. Objectives

Several studies have linked the bisphenols BPA and BPS to metabolic defects such as insulin resistance and T2D. The general aim of this study was to determine the effects of BPA and BPS on skeletal muscle and adipose tissue metabolism. We hypothesized that acute exposure to BPA leads to mitochondrial dysfunction and altered glucose metabolism in the skeletal muscle. Furthermore, we hypothesized that adipose tissue treated with BPA or BPS will have altered glucose metabolism and increased inflammation.

The specific objectives of this study were to:

1. Determine the effects of BPA exposure on muscle mitochondrial function and glucose metabolism and determine if these effects are mediated by the estrogen receptor
2. Determine the effects of BPA and BPS on adipose tissue inflammation and glucose metabolism

3. Methods

3. 1 Effect of acute BPA exposure on L6 muscle cells

3.1.1 Cell culture

Rat L6 myoblasts, kindly provided by Dr. Amira Klip, were grown to 70% confluence in α-minimal essential medium (α-MEM; Wisent, Canada) supplemented with 10% fetal bovine serum (FBS; Wisent, Canada) and 1X antibiotics - antimycotics (AA; Wisent, Canada). Cells were differentiated over a period of 7 days into myotubes in α-MEM with 2% FBS (supplemented with 1X AA). Myotubes were exposed to different concentrations of BPA (1, 10, 10^2, 10^3, 10^4, 10^5 nM) dissolved in dimethyl sulfoxide (DMSO (Sigma-Aldrich, Canada), final concentration 0.1%) or the vehicle (0 nM BPA; 0.1% DMSO) for the last 24 hours of differentiation.

3.1.2 Inhibition of estrogen receptor

To block the estrogen receptor, L6 myotubes were pretreated with 10 nM of the antiestrogen fulvestrant dissolved in DMSO (ICI 182, 780) (Sigma-Aldrich, Canada) for 30 minutes, followed by co-treatment with BPA (10^5 nM) for the last 24 hours of differentiation.

3.1.3 Cell viability

L6 myoblasts were plated on a 96-well plate at 10^4 cells per well. After 7 days of differentiation and BPA treatments, cell viability was assayed spectrophotometrically using lactate dehydrogenase (LDH) Assay Kit (ab65393, Abcam, USA) according to the manufacturer's instructions. Specifically, LDH is a cytoplasmic enzyme that is released into the cell culture supernatant when the cell membrane is damaged, which is a feature of apoptosis (Kumar, Nagarajan and Uchil, 2018). L6 myotubes were exposed to LDH reaction mix for 30 minutes and absorbance was measured at 450 and 650 nm (reference wavelength). Cell viability was calculated

according to the equation [(test sample absorbance - low control absorbance)/(high control absorbance - low control absorbance)]. High control cells were lysed with lysis buffer, according to the manufacturer's instructions. Low control cells were untreated L6 myotubes. Cell cytotoxicity was normalized to 0 nM BPA (vehicle, 0.1% DMSO).

3.1.4 Metabolic Activity

L6 myoblasts were plated on a 96-well plate at 2 x 10^4 cells per well. After 7 days of differentiation and BPA treatments, mitochondrial metabolic activity was assayed spectrophotometrically using PrestoBlue® reagent (Life technologies, Canada) according to manufacturer's instructions. Myotubes were incubated for 30 min in 1X PrestoBlue® reagent and absorbance was measured at 570 and 600 nm (reference wavelength). The PrestoBlue® assay is often used for determining cell cytotoxicity, however, due to the nature of its mechanism, it can also be used for investigating metabolic activity. Specifically, this resazurin-based metabolic assay uses the reducing ability of cells to modify the PrestoBlue reagent (convert resazurin to resorufin), which is quantitatively measured by color change. Resazurin is effectively reduced within mitochondria, therefore, can be used to test for mitochondrial metabolic activity (Sonnaert, Papantoniou, Luyten and Schrooten, 2015).

3.1.5 Glucose uptake

L6 myoblasts were plated at 10^5 cells per well, differentiated, and exposed to BPA or vehicle for 24 hours in 2% FBS α-MEM. L6 glucose uptake was measured as in Klip et al. (1986). Cells were then starved for 3 hours using serum-free α-MEM. Serum contains growth factors and cytokines that contribute to signaling of different pathways, therefore, by serum starving we can accurately measure the effects of insulin on L6 myotubes. Half of the cells were treated with insulin (100 nM) during the last 20 min of serum starvation at 37°C. Cells were washed 3 times and then incubated

for 5 minutes at 37°C in the transport solution HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)-Buffered Saline with 10 μM 2-deoxyglucose and 0.5 μCi/ml [^{3}H] 2-deoxyglucose. For non-specific glucose uptake, L6 cells were incubated for 5 minutes at 37°C with cytochalasin B (10 μM), which was diluted in the transport solution. L6 cells were washed 3 times with ice-cold phosphate-buffered saline (PBS) to stop the reaction. Myotubes were lysed in 0.25 ml of 0.05 M NaOH and 0.2 ml of lysis was mixed vigorously with scintillation liquid (MP biomedicals). This was analyzed by Tri-Carb2910TR scintillation counter (3 minutes/sample) (PerkinElmer, Canada). Protein content was measured using Bradford protein assay in the remaining 0.1 ml of the cell lysate. Glucose transport activity is expressed as pmol of 2-deoxy-glucose taken up/min/μg of total protein.

3.1.6 Oxygen consumption rates (OCR) and extracellular acidification rates (ECAR)

L6 myoblasts were plated on a XF96-well plate (8 x 10^3 cells per well) or a XF24-well plate (3 x 10^4 cells per well) (Agilent, USA). After 7 days of differentiation and treatments, myotubes were washed and incubated for 45 min at 37°C at ambient CO_2, in HCO_3-free assay Dulbecco's Modified Eagle Medium (DMEM) containing 4 mM glutamine, 1 mM sodium pyruvate and 5 mM dextrose (all from Sigma-Aldrich, Canada) (Seahorse assay medium; pH 7.4). Seahorse cartridges were hydrated with XF calibrant, pH 7.4 (Agilent, USA) for approximately 24 hours before experiment at 37 °C without CO_2. Oxygen consumption rates (OCR) and extracellular acidification rates (ECAR; as a marker of glycolysis) were measured using the Seahorse extracellular flux analyzer XF-96 or XF-24 at 37°C (Agilent, USA). Rates were obtained in presence of oligomycin (3 μM), carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP; 2 μM) and antimycin A (4 μM) (all from Sigma-Aldrich, Canada), corresponding to state 4 respiration, maximal respiration (state 3 non-coupled respiration), and non-mitochondrial OCR, respectively, as previously described

(Mauger et al., 2016). Oligomycin inhibits ATP synthase, therefore, remaining OCR is a measurement of OCR due proton leak (state 4 OCR). By inhibiting ATP synthase, oligomycin treatment also allows to measure maximal glycolysis (if mitochondrial ATP synthesis is inhibited, the cells have to shift towards glycolysis to produce ATP). FCCP is a protonophore, and shuttles protons across the inner membrane of the mitochondria, dissipating the proton gradient. Thus, FCCP uncouples the ETC from ATP synthesis, leading to maximal respiration (Tan et al., 2015). Antimycin A inhibits complex III of the ETC, by blocking flow of electrons from cytochrome b to cytochrome c. By inhibiting electron flow, oxygen is not consumed at complex IV, therefore, this allows for the measurement of non-mitochondrial respiration (Tan et al., 2015). The OCR and ECAR parameters included a 3 minute mix time, 3 minute wait time, 3 minute measure time. Following measurements, cells were lysed using 50 µL of 0.05 M NaOH and protein content was assayed using the Bradford protein assay (Biorad, Canada). Mitochondrial OCR and ECAR are expressed per µg of total cellular proteins. Mitochondrial respiration was deduced by subtracting all OCR rates by OCR following antimycin treatment (non-mitochondrial OCR). Since CO_2 produced from the Krebs cycle contributes to the extracellular acidification, ECAR values were corrected for CO_2 production as described by (Mookerjee et al., 2015). It is important to note that maximal ECAR (in the presence of oligomycin) is only an approximation of maximal glycolysis, since it may be limited by ATP demand.

3.1.7 Western blot

L6 myoblasts were plated at 3 x 10^5 cells on a 6-well plate, differentiated, and exposed to BPA or vehicle for 24 hours. L6 myotubes were lysed in ice-cold lysis buffer supplemented with phosphatase and protease inhibitors (20 mM Tris-HCl, 50 nM NaCl, 250 mM sucrose, 1% 100X triton, 50 mM NaF, 5 mM NaP, 1 mM sodium orthovanadate). Samples were sonicated (3 small

pulses at an amplitude 50) on ice and then centrifuged at 13,000 rpm for 10 minutes at 4°C to separate the membrane. Protein content of cell lysates was measured by Bradford (Thermo-fisher) and diluted in 2 X laemmli buffer (9% sodium dodecyl sulfate (SDS), 0.01% bromophenol blue, 50% glycerol, 62.5 mM Tris (0.5 M, pH 6.8), 5% beta-mercaptoethanol). Protein lysates (20 μg) were loaded on 12% polyacrylamide gel electrophoresis, and were run for approximately 15 minutes at 110 V for the stacking gel, and for 45 minutes for the resolving gel. Proteins were transferred to nitrocellulose membranes at room temperature for 1 hour at 110V or overnight at 4°C at 30V. Next, protein bands were detected using a ponceau stain (Thermo-fisher). Membranes were then incubated in a blocking buffer (20 mM Tris buffer saline, 0.05% Tween 20 (TBST) with 5% powdered milk) for 1 hour at room temperature (Thermo-Fisher). Monoclonal anti-glutathione peroxidase 1 (GPx1) (ab108427), monoclonal anti-glutathione peroxidase 4 (GPx4) (ab125066), polyclonal anti-glutaredoxin 2 (Grx2) (ab191292), anti-Akt1 (phospho serine S473) (ab81283), anti-pan-Akt antibody (ab8805), MitoProfile® Total OXPHOS Rodent WB Antibody Cocktail (anti-ATP5a) (MS604) (all from Abcam, Canada), monoclonal anti-catalase (CST-D5N7V, Cell Signaling Technology, Canada), polyclonal anti-α-tubulin (CST-2144, Cell Signaling Technology, Canada), polyclonal superoxide dismutase 2 (SOD2) (sc-30080, Santa Cruz, USA), and monoclonal glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (14C10, Cell Signaling, USA) were used as primary antibodies at a dilution of 1:1000 in TBST with 5% BSA, except for the anti-Akt1 (phospho serine 473) antibody, which was diluted 1:500 in TBST with 5% bovine serum albumin (BSA). Membranes were incubated with selected antibody overnight at 4°C. The secondary antibodies used were anti-mouse (sc-516102) and anti-rabbit (sc-2357) antibodies coupled to horseradish peroxidase (Santa Cruz, USA), diluted 1:5000 TBST with 5% powdered milk, and incubated for 1 hour at room temperature. Proteins were visualized using SuperSignal

West Pico Western Blot Kit (34580, Thermo Scientific, USA) or Clarity Western ECL Substrate (170-5061, Biorad, USA) and imaged using ChemiDoc™ Imager and VisionWorks LS (UVP, USA). Expression of proteins was quantified by densitometry analysis using ImageJ program (National Institutes of Health, USA).

3.2 Effect of BPA and BPS exposure on human adipose tissue

3.2.1 Human participants

Human abdominal subcutaneous tissue was obtained by needle biopsies from 16 healthy individuals (14 women, 2 men, age [21-72], body mass index (BMI) (22.2-32.5 kg/m^2) (Uppsala University Hospital) after administration of local anesthetics (Xylocaine; AstraZeneca, Sweden). Anthropometric and fasting biochemical characteristics are described in Table 3. Fasting blood samples were collected for biochemical analysis of study participants (Uppsala University Hospital). Subjects with T2D, endocrine disorders, cancer or other major illnesses were excluded from the study. The Regional Ethics Review Boards in Uppsala approved the study. All participants gave their written informed consent.

Table 4. Anthropometric and fasting biochemical characteristics of subjects in study.

Variables	
Sex (female/male, n)	14/2
Age (years)	21-72
Body mass index (kg/m^2)	22.2-32.5
Serum insulin levels (mU/l)	9.8 ± 7.3
Plasma glucose levels (mmol/L)	5.3 ± 0.3
Body fat (%)	34.2 ± 7.0
Plasma HDL-cholesterol (mmol/L)	1.44 ± 0.44
Plasma LDL-cholesterol (mmol/L)	2.86 ± 0.78
Plasma triglyceride (mmol/L)	0.96 ± 0.48
HbA1c (mmol/mol)	34.1 ± 3.9

Data are mean ± SD. LDL: low-density lipoprotein; HDL: high-density lipoprotein, HbA1c: glycosylated hemoglobin.

3.2.2 Cell culture

Cells and tissue were cultured in a humidified incubator at 37°C with 5% CO_2. Since bisphenols have been shown to follow a non-monotonic dose response (Acconcia, Pallottini and Marino, 2015), adipose tissue was incubated with environmentally-relevant (1 nM, 10 nM), or supraphysiological concentrations (10^4 nM) of BPA or BPS (Sigma-Aldrich, Canada). Adipose tissue was incubated in low glucose DMEM (1 g/L) (Gibco, Life Technologies), 10% charcoal-stripped FBS (Gibco, Life Technologies), and 1% penicillin-streptomycin (PEST, Invitrogen), in the presence or absence of BPA (1 nM, 10 nM, 10^4 nM), BPS (1 nM, 10 nM, 10^4 nM), dexamethasone (0.3 μM; positive control), or 0.1% DMSO (0 nM; vehicle control) (Sigma-Aldrich) for 24 or 72 hours. After treatment, adipose tissue was either snap frozen in liquid nitrogen or directly used for experimentation.

3.2.3 Cell viability

Adipose tissue was incubated for up to 72 hours in the presence or absence of BPA, BPS, or dexamethasone. Cell viability was measured using the Water Soluble Tetrazolium Salts (WST-1) Assay for Cell Proliferation (Sigma-Aldrich). This involves the cleavage and reduction of WST-1 to formazan which is largely dependent on the glycolytic production of NAD(P)H in viable cells (Ehrke, Steinmeier, Stapelfeldt and Dringen, 2020). Treated adipose tissue was incubated with 10% WST-1 reagent according to manufacturer's protocol. Absorbance was measured at 405 and 630 nm (reference wavelength). Cell viability was calculated using the optical density which was normalized to control (vehicle).

3.2.4 Glucose uptake

Adipose tissue was digested using collagenase A (1 mg/mL) (obtained from *Clostridium histolyticum*, Roche, Manheim, Germany), for 60 min at 37 °C shaking at 105 rpm in Medium 199

(Gibco, Life Technologies, Paisley, UK) supplemented with 6 mM glucose, 4% BSA (Sigma, MO, USA), 150 nM adenosine (Sigma, MO, USA), pH = 7.4. Cells were filtered through 250 μm nylon mesh, and adipocytes fraction was collected and washed four times with Medium 199. Isolated mature adipocytes were subsequently washed four times with 5 min interval with glucose-free Krebs-Ringer bicarbonate medium (KRH) supplemented with 4% BSA, 150 nM adenosine, pH 7.4. Supernatant was isolated, and cells were then subsequently diluted to 6–7% lipocrit with KRH. Isolated adipocytes shaking at 165 rpm at 37 °C were stimulated with 25 or 1000 μIU/ml (0.2 or 6.95 nM) of insulin (physiological and supraphysiological levels) for 15 minutes, followed by incubation with of 14 C-glucose -[^{14}C] glucose (0.26 mCi/L, 0.86 μM, Perkin Elmer, Boston, MA, 161 USA) for 45 minutes. The reaction was stopped by transferring cell suspension to pre-cooled vials. Samples were suspended in 1 ml of silicon fluid (VWR Chemicals, Leuven, Belgium) and centrifuged (5 min, 1000 rpm, 4^{o}C) in order to isolate cell pellet. Radioactivity was measured using Liquid Scintillation Analyser (Perkin Elmer, MA, USA) (5 min, 1 cycle). Cell size was measured under a light microscope as a mean diameter (μm) of consecutively measured 100 adipocytes from the same individual. Cellular glucose uptake was measured according to this formula: Glucose uptake = (cell-associated radioactivity × volume) / (radioactivity of medium × cell number × time).

3.2.5 Western blot

Treated adipose tissue was washed three times with ice-cold PBS and snapped frozen in liquid nitrogen for protein expression analysis. Adipose tissue was homogenized in lysis buffer (25 mM Tris-HCl, 0.5 egtazic acid (EGTA), 25 mM NaCl, 10 mM NaF, 1% NP-40, 100 nM okadaic acid, 1 X protease inhibitor, 1 mM orthovanadate) (Sigma Aldrich, Abcam, Roche). Adipose tissue was incubated at 4 °C for 2 hours rotating to allow separation of protein from adipose tissue. Samples

were centrifuged at 12000g for 15 min at 4 ºC. Clear cell lysate was obtained between the pellet and the fat cake layer using a blunt needle into the eppendorf tube. Protein concentration was measured using bicinchoninic acid assay (BCA) protein assay kit (ThermoFisher). Protein lysates (15 µg) were loaded on 10 % polyacrylamide gel electrophoresis and run for approximately 15 minutes at 90 V for the stacking gel, and for 25 minutes for the resolving gel. Proteins were dry transferred to nitrocellulose membranes at room temperature for 3 minutes. Protein bands were detected using a ponceau stain in 3% Trichloroacetic acid (TCA) (Serva). Membranes were blocked for 1 hour at room temperature with 1x TBS with 1% casein. Anti-Akt (1:1000, Cell Signaling, #9272S), and phospho-Akt (1:1000, Cell Signaling, #9271S) were the primary antibodies used, and were diluted in 1x TBS with 1% casein. The secondary antibodies used were anti-mouse (Cell Signaling, #7076S) and anti-rabbit (Cell Signaling, #7074S) antibodies coupled to horseradish peroxidase (BioRad), diluted 1:5000. Anti-GAPDH was used as a loading control protein (Cell Signaling). Membranes were washed with PBS with 0.05% tween and incubated with appropriate horseradish peroxide conjugated anti-rabbit (Cell Signaling) or anti-mouse (Cell Signaling) secondary antibody. Proteins were visualized using ChemiDoc as described in section 3.1.7. Expression of proteins was quantified by densitometry analysis using ImageJ program (National Institutes of Health, USA).

3.2.6 Gene expression

Treated adipose tissue was washed once with ice-cold PBS and snaped frozen in liquid nitrogen for gene expression analysis. RNA was isolated using RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The concentration and purity of RNA were measured using Nanodrop spectrophotometer (Thermo Scientific). RNA purity was determined using the 260/280 ratio. A ratio of ~2.0 was considered pure for RNA, and values

below this may indicate the presence of protein, phenol or other contaminants. RNA (400 ng) was then reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher Scientific, CA, USA). The data were calculated using the 2-delta Ct (cycle threshold) method. The results were normalized using the housekeeper gene 18S ribosomal RNA (18S rRNA). Gene expression of markers of inflammation (IL-6, IL-1B, TNFa, IL-33), fatty acid transport (FABP4), glucose transport (GLUT4/SLC2A4), adipokines (adiponectin, leptin)) measurement was performed with the QuantStudio 3 System (Thermo Fisher Scientific, MA, USA) using TaqMan assay probes listed in Table 5 (Thermo Fisher Scientific, MA, USA). All samples were run in duplicates.

3.3 Statistical Analysis

Data are presented as means ± SEM or SD. Unpaired 2-sided t tests, one way analysis of variance (ANOVA) with Bonferroni or Dunnet's correction with repeated measures or mixed-effects model analysis as appropriate, and two-way ANOVA with a Fisher PLSD post hoc test and Tukey's multiple comparisons test were used to assess statistical differences with Statview 5.0 Software (SAS Institute, USA) and GraphPad Prism version 6.0e (La Jolla, USA) or GraphPad Prism version 8.4.3 (La Jolla, USA). Values with $p < 0.05$ were considered significant.

Table 5. Taqman probes for genes used in this study

Gene	Taqman probe
Adiponectin	Hs00605917_m1
Fatty acid binding protein 4 (FABP4)	Hs01086177_m1
Interleukin 1 beta (IL1B)	Hs01555410_m1
Interleukin 6 (IL6)	Hs00985639_m1
Interleukin 33 (IL33)	Hs04931857_m1
GLUT4 (SLC2A4)	Hs00168966_m1
Tumor necrosis factor (TNF)	Hs00174128_m1

4. Results

4.1 Effect of acute BPA exposure on L6 muscle cells

The following results (section 4.1) have been reprinted from Elsevier. © 2020 Molecular and Cellular Endocrinology: The effects of acute BPA exposure on skeletal muscle mitochondrial function and glucose metabolism). This paper is attached as an appendix.

4.1.1 Acute BPA exposure does not alter cell viability of L6 myotubes

Acute BPA treatment at concentrations used in our study has been shown to alter cell viability in certain cell types, such as Sertoli cells; however, have no effect on cell viability in others, such as adipocytes (Zhang et al., 2017; Desai et al., 2018). Therefore, we wanted to evaluate the effect of BPA exposure on L6 myotube viability. The release of LDH from cells is a marker of cell cytotoxicity; therefore, LDH was spectrophotometrically measured in L6 myotubes exposed to BPA (1 nM –10^5 nM) for 24 hours. As shown in Fig. 5, BPA exposure from 1 nM to 10^5 nM for 24 hours did not significantly affect L6 myotube viability ($p>0.05$).

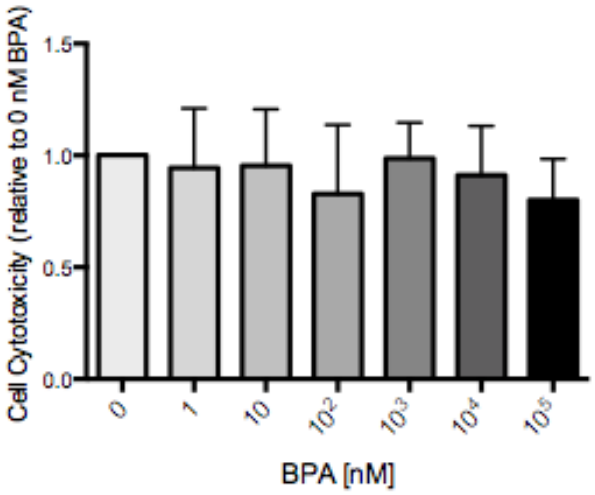

Figure 5. The effect of 24h BPA exposure on L6 cell viability. Cell cytotoxicity measured by lactate dehydrogenase (LDH) release from L6 myotubes following BPA exposure for 24 hours (1 nM – 10^5 nM) relative to the vehicle control (0 nM BPA; 0.1% DMSO). Cell cytotoxicity describes the ratio of LDH released between the test sample (BPA-treated cells) and a positive control (lysed cells). n=3, each independent experiment was done in triplicate. Data represent mean ± SEM. *P < 0.05 (one-way ANOVA). Figure from Ahmed et. al. (2020).

4.1.2 Acute exposure to high concentrations of BPA alters mitochondrial function and metabolic activity of L6 myotubes

To determine whether acute BPA exposure alters mitochondrial function in skeletal muscle cells, L6 myotubes were exposed to different concentrations of BPA for 24 hours and OCR was measured in basal conditions and following oligomycin and FCCP treatment to assess OCR due to proton leak and maximal mitochondrial capacity, respectively (Fig. 6). BPA exposure between 1 nM and 10^4 nM did not significantly alter basal respiration in the presence of glucose, pyruvate, and glutamine as substrates; however, basal respiration was significantly decreased at 10^5 nM of BPA (Fig. 6A and B) ($p<0.05$). The trace OCR shows respiration over a time period (Fig. 6B). L6 myotubes exposed to 10^5 nM of BPA also showed an increased mitochondrial proton leak whereas maximal respiration was reduced compared to the control group (Fig. 6A) ($p<0.05$). The percentage of basal OCR due to proton leak was increased in L6 myotubes exposed to 10^5 nM of BPA (Fig. 6C) ($p<0.05$). Mitochondrial metabolic activity (reducing capacity of mitochondria) was also lower in L6 myotubes exposed to 10^5 nM of BPA (Fig. 6D) ($p<0.05$). To determine if the decreased mitochondrial function with high BPA exposure was the result of altered levels of ATP synthase, we measured the protein level of ATP synthase. ATP synthase (ATP5a) levels were not significantly altered by BPA treatment (Fig. 6E and F).

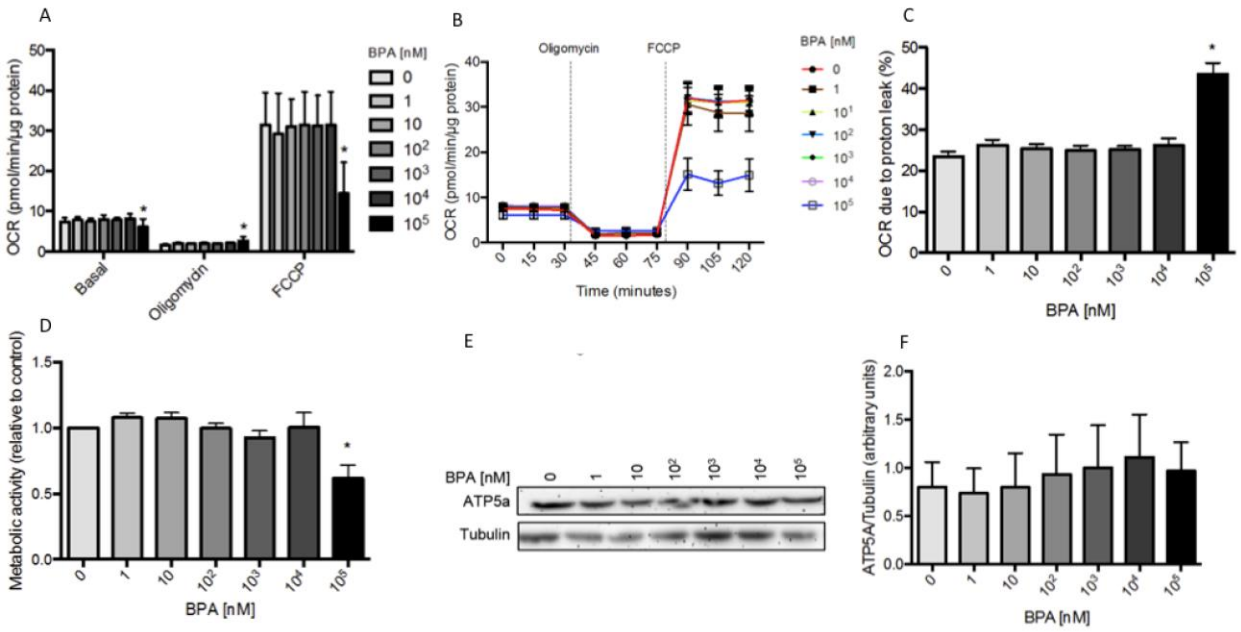

Figure 6. The effects of 24-hour BPA exposure on mitochondrial function and metabolic activity in L6 myotubes. L6 myotubes were exposed to the vehicle (0,1% DMSO) or different concentrations of BPA (1 nM – 10^5 nM) for 24 hours. (A) OCR and (B) trace OCR was first measured followed by treatments with: oligomycin (3 µM) to assay for proton leak, and FCCP (2 µM) to assay maximal OCR. n=6, each independent experiment was done in 4-6 replicates. (C) Percent of basal OCR due to proton leak (D) Metabolic activity measured by PrestoBlue® assay of L6 myotubes exposed to BPA or vehicle (DMSO) for 24h. n=3, each independent experiment was done in 4-6 replicates. (E and F) Protein levels of ATP5a (ATP synthase) in L6 myotubes treated with BPA or vehicle control (DMSO) for 24 hours (n=3 independent experiments). Left panel: representative western blot of ATP5a. Right panel: quantification of ATP5a by density analysis. α-tubulin was used as a loading control. (A-F) Data represent mean ± SEM. *$p < 0.05$ compared to vehicle (one-way ANOVA). Figures adapted from Ahmed et. al. (2020).

4.1.3 Exposure to high concentrations of BPA increases glucose metabolism in L6 myotubes

Extracellular acidification rate was measured as an indicator of glycolytic function in the resting state and by blocking mitochondrial ATP-synthase by oligomycin as an indirect measurement of maximal ATP production through anaerobic glycolysis (Fig. 7). Exposure to BPA between 1 nM and 10^4 nM did not alter glycolytic rate in L6 myotubes (Fig. 7A and B) ($p>0.05$); however, exposure to 10^5 nM of BPA significantly increased resting and maximal glycolysis in L6 myotubes (Fig. 7A) ($p<0.05$). The trace ECAR shows the glycolytic rate over a time period (Fig. 7B). Next, basal and insulin-stimulated glucose uptake was measured in L6 myotubes exposed to BPA. Cells exposed to 10^5 nM of BPA had increased uptake of 2-deoxyglucose under both basal and insulin-stimulated conditions ($p<0.05$) (Fig. 7C), with no effect on insulin sensitivity (measured as the fold-increase in 2-deoxyglucose uptake in response to insulin) (Fig. 7D). To investigate the effects of BPA exposure on the insulin-signaling pathway, phosphorylation of the serine/threonine kinase Akt, a key protein in the insulin signaling pathway, was measured by western blot (Fig. 7E and F). Insulin-stimulated L6 myotubes exposed to 10^5 nM of BPA also showed significantly increased phosphorylation of Akt ($p<0.05$) (Fig. 7E and F).

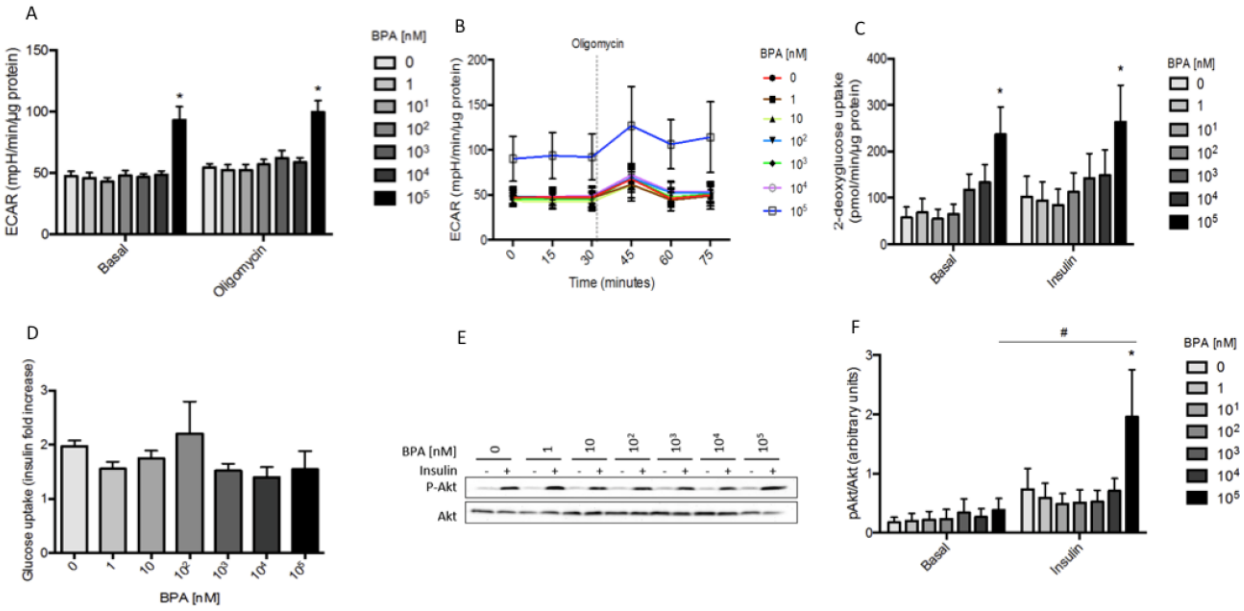

Figure 7. The effects of 24-hour BPA treatment on glucose metabolism in L6 myotubes. Resting and maximal ECAR (A) and trace ECAR (minutes) (B) of L6 myotubes exposed for 24 hours to BPA (1 nM – 10^5 nM) or vehicle (0.1% DMSO). n=6, each independent experiment was done in 4-6 replicates (one-way ANOVA). (C) Uptake of 2-deoxyglucose and (D) fold-increase in glucose uptake in response to insulin treatment measured in L6 myotubes exposed to BPA or vehicle (0.1 % DMSO). L6 myotubes were exposed to insulin (100 nM) for 20 min. n=5, each independent experiment was done in triplicate. (E and F) Ratio of phospho-Akt and total Akt in L6 myotubes exposed for 24 hours to BPA or vehicle (0.1% DMSO). L6 myotubes were subsequently exposed to insulin (100 nM) for 20 min. Left panel: representative western blot of phospho-Akt and Akt. Right panel: quantification of phospho-Akt/Akt by density analysis. n=3 independent experiments. A-D. * $p<0.05$ compared to all other BPA concentrations in the same condition. # for the main effect of insulin (two-way ANOVA). Data represent mean ± SEM. Figures adapted from Ahmed et. al. (2020).

4.1.4 The effects of ER antagonist ICI on glucose uptake and mitochondrial function in L6 myotubes exposed to BPA

To explore the mechanism by which high concentrations of BPA exert altered glucose metabolism and mitochondrial function, we used the specific ER antagonist ICI to inhibit the ER before measuring glucose uptake, mitochondrial function and glycolytic rates (Fig. 8 A-D). Specifically, ICI binds to ERα, leading to a conformational change and accelerated degradation of the receptor (Moverare-Skrtic et al., 2014). Co-incubation of BPA (10^5 nM) and ICI (10 nM) reversed BPA-induced alteration of glucose uptake in both the absence and presence of insulin ($p<0.05$) (Fig. 8A), however, BPA-induced alterations of OCR and ECAR were not reversed following co-incubation with ICI (Fig. 8B-D).

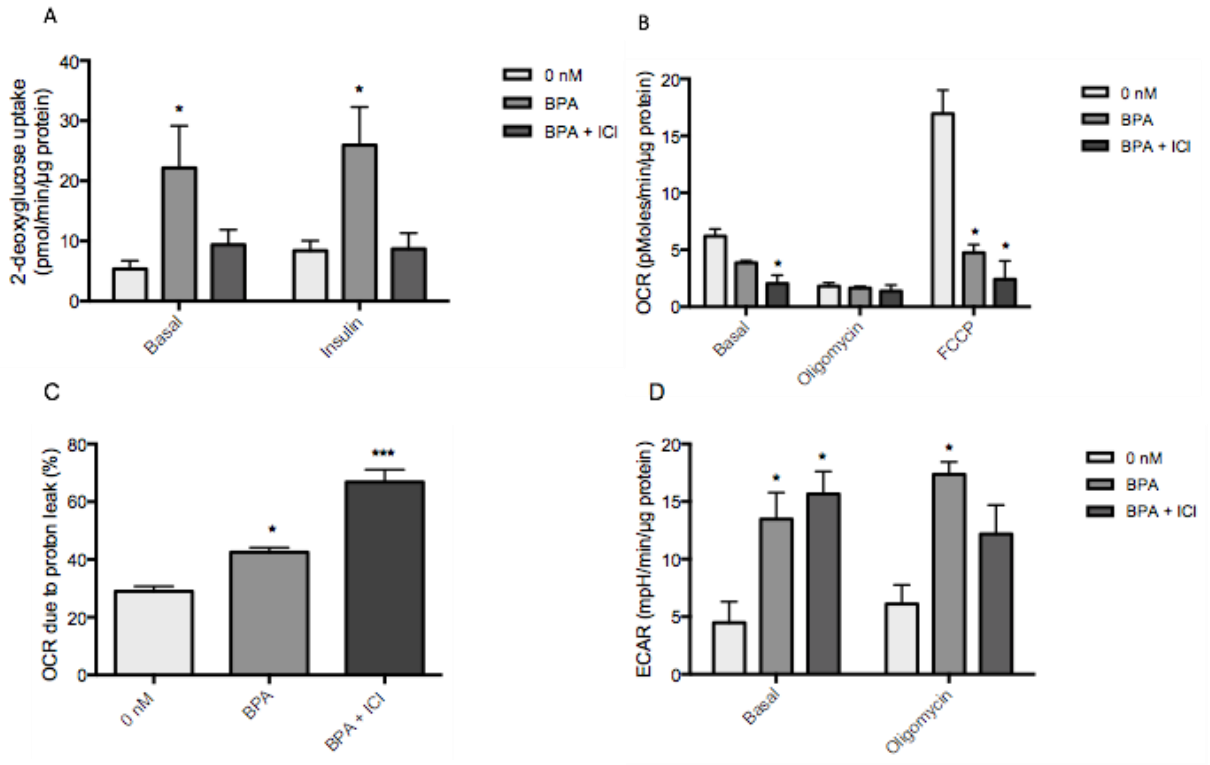

Figure 8. The effects of ICI on BPA-induced glucose uptake and mitochondrial dysfunction in L6 myotubes. L6 myotubes were treated with vehicle (0.1% DMSO), BPA (10^5 nM), or BPA (10^5 nM) and ICI (10 nM) during the last 24 hours of differentiation. (A) Uptake of 2-deoxyglucose in L6 myotubes treated ± insulin (100 nM) for 20 minutes. (B) Basal OCR was first measured followed by treatments with: oligomycin (3 µM) to assay for proton leak, and FCCP (2 µM) to assay maximal OCR. n=4, each independent experiment was done in 3-5 replicates. (C) Percent of basal OCR due to proton leak (n=4). (D) Basal and maximal (oligomycin-treated) ECAR. Data represent mean ± SEM. *$p < 0.05$ compared to vehicle. A-D. * $p<0.05$ compared to all other BPA concentrations in the same condition. (two-way ANOVA). Data represent mean ± SEM. Figures from Ahmed et. al. (2020).

4.1.5 Effects of BPA exposure on oxidative stress markers in L6 myotubes

Mitochondrial dysfunction can be the cause or a consequence of oxidative stress. Since we showed that L6 myocytes exposed to BPA have reduced mitochondrial function, we wanted to investigate if there was alteration in protein levels of oxidative stress markers in these cells. To determine the effects of 24-hour BPA exposure on oxidative stress in L6 myotubes, the levels of the oxidative stress markers GPx1, GPx4, Grx2, SOD2, and catalase were measured. There was no significant change in the protein level of GPx1, GPx4, Grx2, catalase, and SOD2, however, there was an increased trend for SOD2 (Fig. 9A-F).

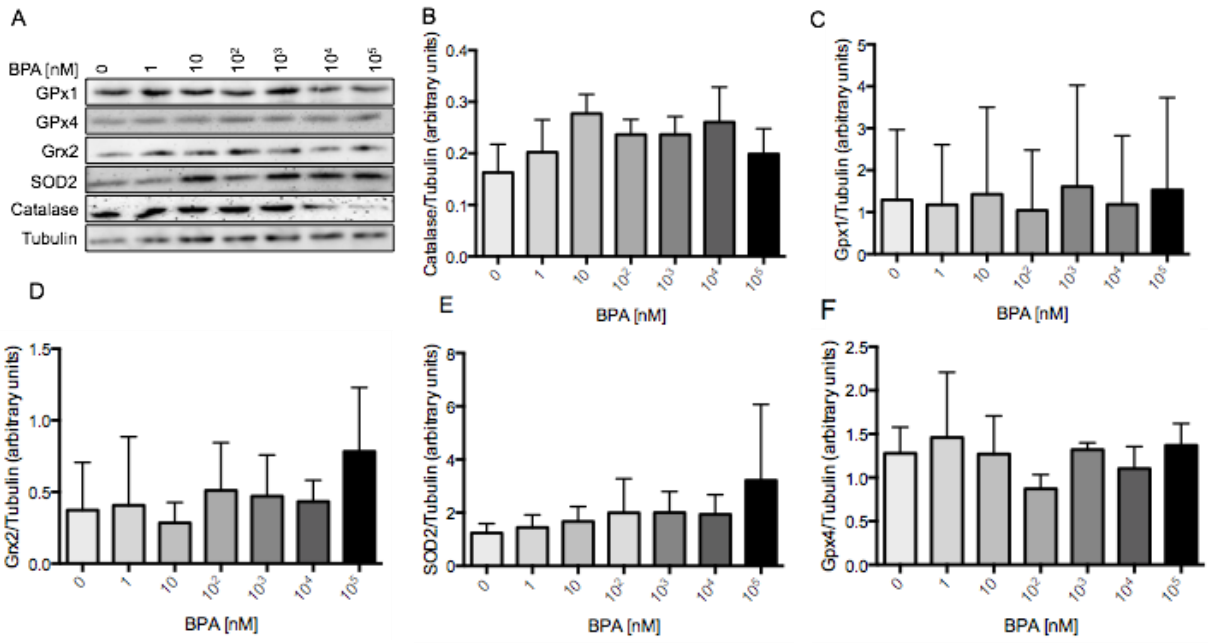

Figure 9. The effects of 24-hour BPA treatment on oxidative stress markers in L6 myotubes. (A) Representative western blot of the oxidative stress markers GPx1 and 4, Grx2, SOD2, and catalase. Tubulin was used as a loading control. (B-F) Quantification of catalase (B), GPx1 (C), Grx2 (D), SOD2 (E), and GPx4 (F) by density analysis (n=3 independent experiments). Data represent mean ± SEM (one-way ANOVA). Figure from Ahmed et. al. (2020).

4.2 Effect of BPA and BPS exposure on human adipose tissue

4.2.1 The effects of BPA or BPS on human adipose tissue cell viability

Next, we evaluated the effects of BPA, BPS, or dexamethasone (positive control) exposure on adipose tissue cell viability. The reduction of the stable tetrazolium salt WST-1 to formazan, which is an indication of cell proliferation and viability, was measured spectrophotometrically in adipose tissue that was treated for up to 72 hours with BPA, BPS or dexamethasone. We found that BPA, BPS, or dexamethasone had no effect on cell viability in adipose tissue (Fig. 10).

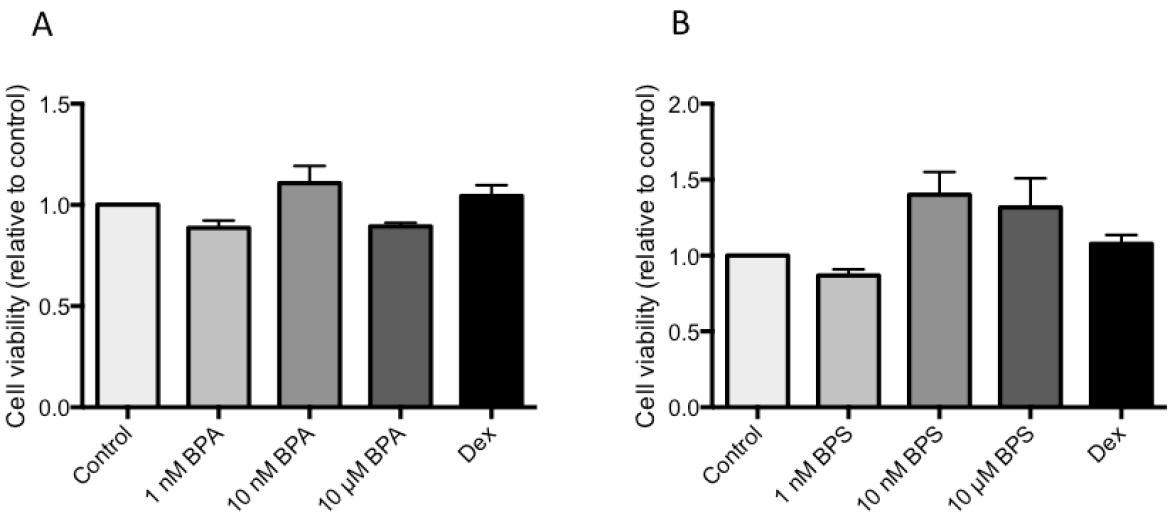

Figure 10. The effects of BPA and BPS exposure on cell viability in human abdominal subcutaneous adipose tissue. Adipose tissue from human subcutaneous adipose tissue biopsies were incubated ex vivo with BPA or BPS (1 nM, 10 nM and 10^4 nM) for up to 72 hours and compared to the vehicle control (0.1% DMSO) and the positive control dexamethasone (Dex, 0.3 µM). Cell viability was measured by cleavage of the tetrazolium salt to formazan by cellular mitochondrial dehydrogenase (proportional to live cells). n=3-4, each independent experiment was done in duplicate. Data represent mean ± SEM. (one-way ANOVA).

4.2.2 The effects of 24 and 72 hour BPA and BPS exposure on adipokine gene expression in human adipose tissue

The effects of BPA and BPS exposure on expression of proinflammatory cytokines, adipokines, and receptor levels was measured in adipose tissue following 24 and 72 hour treatments (Table 6). Following 24 hour BPA treatment, there was a reduction in the expression of the proinflammatory cytokines *IL-6* (1 nM and 10 nM), *IL-1B* (10 nM), and *TNFA* (1 nM) ($p<0.05$). The proinflammatory cytokine *IL-33* was not affected by BPA or BPS treatment at 24 hours (Table 3). Exposure to BPA for 24 hours reduced gene expression of the adipokines *ADIPOQ* (10 nM) and FABP4 (10 nM) ($p<0.05$), with no effect on *LEP* expression (Table 3). *FABP4* was also reduced when exposed to 1 nM of BPS for 24 hours ($p<0.05$) (Table 3). Gene expression of the receptors *ESR1* and *ESR2* were unaffected by 24 hour BPA and BPS exposure. Adipose tissue treated with BPA or BPS for 72 hours had no effects on gene expression of proinflammatory cytokines, adipokines, and estrogen receptors (*ESR1* and *ESR2*) (Table 6).

Table 6. The effects of 24 or 72 hour BPA and BPS exposure gene expression in human subcutaneous adipose tissue.

	BPA			BPS			Dex
Gene	1 nM	10 nM	10^4 nM	1 nM	10 nM	10^4 nM	0.3 μM
24 hours							
Proinflammatory cytokines							
IL-6	0.72 ± 0.08	**0.56± 0.21***	**0.51 ± 0.11***	1.08 ± 0.15	1.41 ± 0.44	1.86 ± 0.76	**0.30 ± 0.09***
IL-1B	0.74 ± 0.20	**0.58 ± 0.13***	0.55 ± 0.18	1.04 ± 0.27	1.06 ± 0.23	1.22 ± 0.13	**0.09 ± 0.02***
TNFA	**0.80 ± 0.04***	0.84 ± 0.14	0.68 ± 0.16	1.05 ± 0.14	1.12 ± 0.41	0.94 ± 0.17	**0.43 ± 0.10***
IL-33	0.86± 0.04	0.82 ± 0.15	0.73 ± 0.13	0.90± 0.09	1.60 ± 0.47	1.13 ± 0.13	**0.07 ± 0.02***
Adipokines							
ADIPOQ	0.69 ± 0.10	**0.65 ± 0.12***	0.75 ± 0.15	0.81 ± 0.07	0.92 ± 0.16	1.11 ± 0.28	0.94 ± 0.15
FABP4	0.77 ± 0.09	**0.59 ± 0.11***	0.74 ± 0.20	**0.76 ± 0.05***	0.73 ± 0.09	1.06 ± 0.16	0.95 ± 0.15
LEP	0.84 ± 0.05	0.86 ± 0.14	0.82 ± 0.16	1.15 ± 0.11	1.32 ± 0.29	1.03 ± 0.10	**3.07 ± 0.73***
Receptors							
ESR1	0.91 ± 0.08	0.82 ± 0.13	0.79 ± 0.12	0.97 ± 0.08	1.10 ±0.18	1.11 ± 0.17	0.98 ± 0.18
ESR2	0.94 ± 0.14	0.77 ± 0.12	0.77 ± 0.12	1.02 ± 0.19	1.02 ± 0.17	1.01 ± 0.17	**1.72 ± 0.29***
72 hours							
Proinflammatory cytokines							
IL-6	1.53 ± 0.15	1.66 ± 0.31	1.27 ± 0.20	5.25 ± 2.0	3.93 ± 0.96	3.74 ± 1.28	1.20 ± 0.65
IL-1B	1.13± 0.39	0.91 ± 0.19	0.94 ± 0.28	1.73 ± 0.04	2.28 ± 0.73	2.93 ± 0.97	**0.64 ± 0.02***
TNF	0.78 ± 0.26	0.88 ± 0.30	0.95 ± 0.32	0.87 ± 0.20	1.89 ± 0.62	0.89 ± 0.20	**0.44 ± 0.11***
IL-33	1.22 ± 0.16	1.27 ± 0.25	1.0 ± 0.17	1.87 ± 0.48	2.98 ± 0.88	2.68 ± 1.19	0.93 ± 0.51
Adipokines							
ADIPOQ	1.23 ± 0.18	1.87 ± 0.67	1.55 ± 0.22	1.47 ± 0.31	1.79 ± 0.46	4.45 ± 1.88	**2.36 ± 1.22***
FABP4	1.04 ± 0.21	2.27 ± 1.04	1.17 ± 0.29	1.79 ± 0.54	2.40 ± 0.77	5.62 ± 2.63	3.70 ± 2.80
LEP	1.02 ± 0.26	1.01 ± 0.17	1.04 ± 0.30	1.41 ± 0.52	1.38 ± 0.21	0.51 ± 0.13	2.15 ± 0.54
Receptors							
ESR1	0.93 ± 0.14	0.87 ± 0.14	0.89 ± 0.22	1.00 ± 0.14	1.07 ± 0.11	1.21 ± 0.11	0.82 ± 0.16
ESR2	0.93 ± 0.22	1.03 ± 0.21	0.95 ± 0.20	1.29 ± 0.24	1.11 ± 0.11	2.05 ± 0.48	2.30 ± 0.61

IL-6 - Interleukin-6 (24 hours: n=8, 72 hours: n=4); *IL-1B* - Interleukin-1β (24 hours: n=7, 72 hours: n=5); *TNFA* - Tumor Necrosis Factor α (24 hours: n=6, 72 hours: n=5); *IL-33* - Interleukin 33 (24 hours n=7, 72 hours: n=5); *ADIPOQ* - Adiponectin (24 hours: n=8, 72 hours: n=5); *FABP4* - Fatty acid binding protein 4 (24 hours: n=8, 72 hours: n=5); *LEP* - Leptin (n=8, 72 hours: n=5); *ESR1* - Estrogen Receptor 1 (24 hours: n=8, 72 hours: n=8); *ESR2* - Estrogen Receptor 2 (24 hours: n=8, 72 hours: n=6). Human subcutaneous adipose tissue incubated with BPA, BPS, dexamethasone (Dex, positive control), or DMSO (vehicle control), for 24 or 72 hours. 18S was used as the housekeeping gene. Gene expression data is represented as a fold change to vehicle control. Relative expression was calculated as 2^(-ΔΔCt). Data represent mean ± SEM. n=3-4, *$p < 0.05$ (one-way ANOVA).

Table 7. Summary of genes affected by 24 hour BPA, BPS, and dexamethasone treatment.

Gene	BPA			BPS			Dex
	1 nM	10 nM	10^4 nM	1 nM	10 nM	10^4 nM	(0.3 μM)
Proinflammatory							
IL-6	N.S.	↓	↓	N.S.	N.S.	N.S.	↓
IL-1B	N.S.	↓	N.S.	N.S.	N.S.	N.S.	↓
TNFA	↓	N.S.	N.S.	N.S.	N.S.	N.S.	↓
IL-33	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	↓
Adipokines							
ADIPOQ	N.S.	↓	N.S.	N.S.	N.S.	N.S.	↓
FABP4	N.S.	↓	N.S.	N.S.	↓	N.S.	↓

IL-6 - Interleukin-6 (n=8); *IL-1B* - Interleukin-1β (n=7); *TNFA* - Tumor Necrosis Factor α (n=6); *IL-33* - Interleukin 33 (n=7); *ADIPOQ* – Adiponectin (n=8); *FABP4* - Fatty acid binding protein 4 (n=8). 18S was used as the housekeeping gene. N.S. no significance. Gene expression data is represented as a fold change to vehicle control. Relative expression was calculated as 2^(-ΔΔCt). Data represent mean ± SEM. n=3-4, *p < 0.05 (one-way ANOVA).

4.2.3 The effects of BPA exposure on maximal glucose uptake in human adipose tissue

Since 72 hour bisphenol exposure had no apparent effect on adipose tissue gene expression, in addition to nearly no effect with 24 hour BPS exposure, we focused on the effects of 24 hour BPA exposure. To determine the effects of BPA on glucose metabolism, we performed a radioactive 14C-glucose uptake assay in human adipose tissue exposed to vehicle control (0.1% DMSO), BPA (10 nM or 10^4 nM), or dexamethasone, for 24 hours. Following BPA incubation with adipose tissue, adipocytes were isolated and basal and insulin-stimulated glucose uptake was measured. Maximal glucose uptake (1000 μU/mL insulin) was significantly reduced in adipocytes treated with 10 and 10^4 nM BPA, and dexamethasone (Fig. 11A). Basal adipocytes treated with 25 and 1000 μU/mL had significantly increased glucose uptake (Fig. 11B), however, there was no insulin effect (25 and 1000 μU/mL) in adipose tissue treated with BPA (10 and 10^4 nM) or dexamethasone (Fig. 11B).

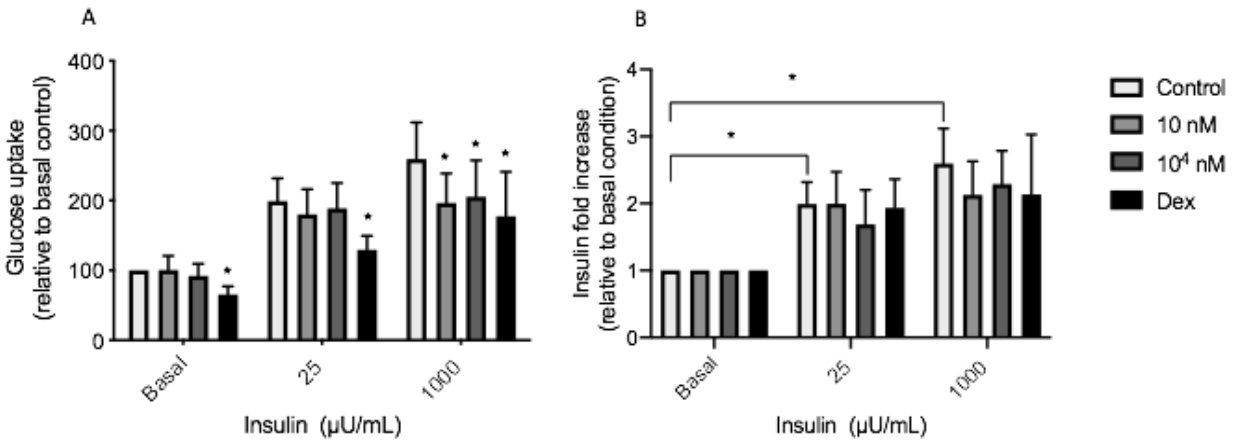

Figure 11. The effects of 24 hour BPA and BPS treatment on glucose uptake in adipocytes from human subcutaneous abdominal adipose tissue. (A) Uptake of radioactive [^{14}C] 2-deoxyglucose and (B) insulin-fold increase in isolated adipocytes from adipose tissue treated with vehicle (0.1% DMSO), BPA (10 nM, 10^4 nM), or dexamethasone (Dex, 0.3 µM, positive control). Adipocytes were exposed to (25 µU/mL, 1000 µU/mL) insulin for 1 hour. For fold increase, basal conditions from each treatment were normalized to 1. n=7, each independent experiment was done in duplicate or triplicate. *$p < 0.05$ compared to all other BPA concentrations in the same condition. (A-D) Data represent mean ± SEM. *$p < 0.05$ (one-way ANOVA).

4.2.5 The effects of 24 hour BPA treatment on the insulin signaling pathway in human adipose tissue

Next, we wanted to determine whether the inhibitory effects of BPA on insulin-stimulated glucose uptake could be due to effects of key protein involved in the insulin signalling. Expression level of glucose transporters GLUT4 and GLUT1 was measured in adipose tissue treated with BPA for 24 hours. BPA exposure did not result in change in GLUT4 or GLUT1 mRNA levels (Fig. 12A and B). We then looked at whether BPA exposure had an effect on key regulators of the insulin signaling pathway in adipose tissue, by measuring expression levels of *AKT* and *IRS1*. We found no changes in gene expression of *AKT* or *IRS1* in adipose tissue exposed to BPA (Fig. 12C and D). Protein levels of GLUT4 were measured to determine if BPA altered protein levels, however, we found no significant effect (Fig. 12E). Next, we measured levels of phosphorylated and total protein levels of Akt. We found that 24 hour BPA exposure did no effect on phosphorylation of Akt in adipose tissue treated with BPA (Fig. 12F).

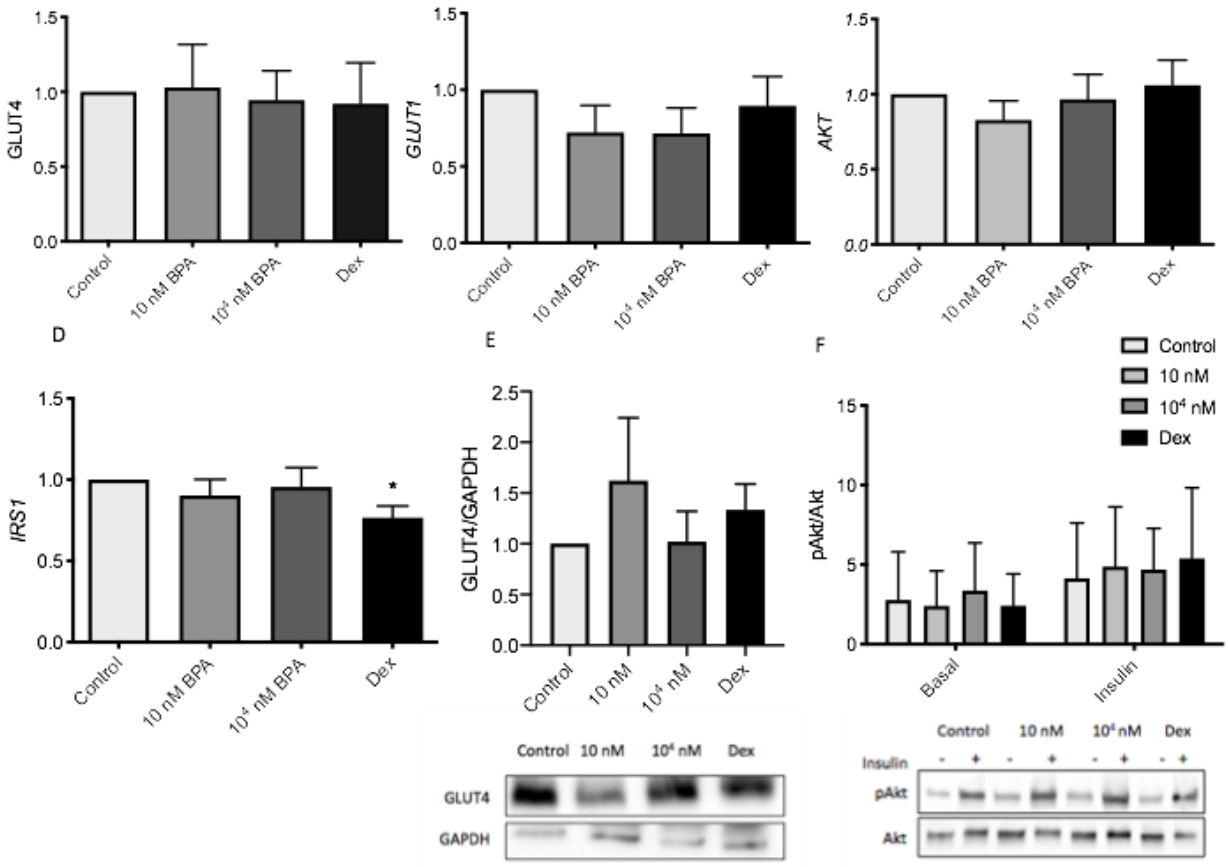

Figure 12. The effects of 24 hour BPA treatment on the insulin signaling pathway from human subcutaneous abdominal adipose tissue. Gene expression of (A) GLUT4 and (B) GLUT1 (C) AKT (D) IRS1 in adipose tissue treated with vehicle (DMSO), BPA (1 nM - 10^4 nM), or dexamethasone (Dex), measured by quantitative PCR (qPCR). n=3, independent experiments. (E) Protein level of GLUT4. Top panel: quantification of GLUT4 by density analysis. Bottom panel: representative Western blot. (F) Ratio of phosphorylated and total Akt in adipose tissue treated with DMSO, BPA (10 nM, 10^4 nM) or dexamethasone (Dex). Adipose tissue was exposed to insulin (1000 µU/mL) for 15 min. Top panel: quantification of phospho-Akt/Akt by density analysis. Bottom panel: representative Western blot. n = 3 independent experiments. (A-F) Data represent mean ± SEM.

5. Discussion

Recent research has been focused on the role of environmental pollutants such as bisphenols on the development of T2D (Jeon, Ha and Kim, 2015), however, the exact mechanisms by which bisphenols exert their harmful effects on metabolism have not been completely elucidated. Since both skeletal muscle and adipose tissue play an important role on whole-body glucose metabolism, we investigated the effects of bisphenol exposure on skeletal muscle and adipose tissue metabolism. Specifically, we wanted to study the effects of acute BPA exposure on skeletal muscle mitochondrial function and glucose metabolism. Since the adipose tissue is an endocrine organ that communicates with peripheral tissue such as the skeletal muscle to alter metabolic function, we also investigated the effects of BPA and BPS exposure on human adipose tissue inflammatory marker expression and glucose metabolism. In general, our study found no effects of environmentally-relevant concentrations of BPA on skeletal muscle mitochondrial function and glucose metabolism. Furthermore, adipose tissue treated with BPA showed changes in pro-inflammatory gene expression and altered glucose metabolism. Our objectives and results will be discussed in further detail below.

5.1 The effects of bisphenols on skeletal muscle mitochondrial function and glucose metabolism

Skeletal muscle mitochondrial dysfunction has been linked to the development of insulin resistance and T2D. Although studies have demonstrated a link between BPA exposure and muscle insulin resistance, it was unknown whether BPA could alter muscle mitochondrial function, and in turn, if this contributes to insulin resistance. Therefore, in this study, we investigated the effects of acute BPA exposure on muscle mitochondrial function, by measuring mitochondrial respiration, mitochondrial complex content, and mitochondrial oxidative stress markers.

First, we wanted to determine the effects of BPA on L6 myotube respiration by measuring the OCR of L6 myotubes treated with increasing concentrations of BPA for 24 hours. We found that 24 hour exposure of BPA at environmentally-relevant concentrations did not affect mitochondrial function in these cells. Interestingly, L6 myotubes acutely exposed to supraphysiological concentrations of BPA (10^5 nM) have reduced basal and maximal mitochondrial OCR. This was consistent with the metabolic assay we performed, which showed reduced ability of the mitochondria to convert resazurin to resorufin. This was similar to a *in vitro* study, where 24 hour BPA exposure induced depolarization of the mitochondrial membrane potential, inhibition of the mitochondrial respiratory chain activity, and reduction of ATP production in intestinal goblet cells (LS174T cells) (Zhao et al., 2019). Furthermore, we found that at high concentrations of BPA (10^5 nM) proton leak was increased in L6 myotubes. This aligns with patients with T2D, which have been shown to have increased proton leak, and reduced basal and maximal respiration (Phielix et al., 2008; Aguer et al., 2013). Specifically, mitochondrial proton leak can reduce mitochondrial membrane potential, which reduces ROS (Mailloux and Harper, 2011). Mitochondrial UCPs are anion carrier proteins and play an important role in reducing ROS production (Mailloux and Harper, 2011). Therefore, it is possible that UCP3, which is the skeletal muscle isoform, may be increased by 24 hour BPA exposure and may explain increased proton leak (Mailloux and Harper, 2011). In an *in vivo* study, BPA was shown to have effects on mitochondrial function at the tolerable daily intake concentration of 50 μg/kg/day (Wang, Zhao and Ji, 2019). Specifically, CD-1 mice exposed to dietary BPA (50 μg/kg/day) had reduced mitochondrial encoded genes and copy number in the liver and colon tissue (Wang, Zhao and Ji, 2019). Furthermore, they showed that in colon tissue, mitochondrial complex IV and V activity was reduced, whereas I-V was reduced in liver tissue (Wang, Zhao and Ji, 2019). It is possible that *in vivo* acute BPA exposure at low

concentrations can alter mitochondrial function in certain organs such as the colon and liver, but not in the skeletal muscle.

Changes in muscle mitochondrial OXPHOS proteins such as ATP synthase have been shown to be modified in obesity and T2D. Specifically, Formentini et al. (2017) showed that levels of ATP synthase appear to be downregulated in human skeletal muscle from obese individuals. Furthermore, they showed that ATP synthase activity was reduced in these cells, and the ATPase inhibitory factor 1 (IF1) was overexpressed in individuals with obesity. We therefore measured ATP5a levels in L6 myotubes, and found that at both environmentally-relevant and supraphysiological levels of BPA, ATP5a protein levels were unaffected. Therefore, it is unlikely that the mitochondrial dysfunction at 10^5 nM of BPA was due to reduced levels of ATP5a. Although we did not find a change in ATP5a, it is possible that ATP synthase activity may have been affected by BPA treatment. Therefore, future studies investigating the effects of BPA on ATP synthase activity may provide insight to the mechanism by which BPA induces mitochondrial dysfunction at high concentrations.

Recent studies have indicated that ROS production is linked to many chronic diseases including T2D. Increased oxidative stress is often the result of mitochondrial dysfunction, and this can lead to further mitochondrial damage and eventually result in mitophagy (Montgomery and Turner, 2015). Furthermore, mitochondrial mitophagy and reduced mitochondrial number can result in an overall reduction in mitochondrial respiration. Since the mitochondria are a major source of ROS, we measured protein levels of ROS markers. In our study, we found no effect of BPA exposure on protein levels of GPx1, GPx4, Grx2, catalase, and SOD2. It is possible that 24 hour BPA exposure is not long enough to induce changes in protein levels of these proteins. Proton leak, measured after inhibition of ATP synthase by oligomycin, was increased when cells were exposed to 10^5 nM

of BPA. As previously mentioned, mitochondrial proton leak is believed to decrease mitochondrial ROS production to protect cells from oxidative stress; therefore, it is possible that any ROS production following BPA exposure was counteracted by increased proton leak, possibly due to increased UCP3 (Mailloux and Harper, 2011). In addition, although we measured the protein levels of ROS detoxification enzymes, there could be modifications to the activity of these enzymes. It is also possible that in the skeletal muscle 24 hour BPA exposure was not long enough to cause ROS in skeletal muscle cells; therefore, future studies should investigate the effect of chronic BPA exposure to determine whether a longer exposure affects ROS levels. Moreover, ROS increase by BPA has been reported in different cell types such as human β cells, however, differences in ROS generation appear to be due to differences in treatment duration, cytotoxicity, and concentrations of BPA, and background contamination levels of BPA (ie. from cell culture flasks) (Gassman, 2017; Meli et al., 2020). Directly measuring ROS would confirm whether ROS was produced by BPA exposure, which should be done in future studies.

The skeletal muscle is rich in mitochondria, and thus energy production is strongly reliant on oxidative phosphorylation for energy production (Kelley, He, Menshikova and Ritov, 2002). BPA-induced perturbations of mitochondrial function, and possibly reduced energy production, at high concentrations may affect glucose metabolism. We investigated the effects of acute BPA exposure on glucose metabolism of skeletal muscle. First, we measured the glycolytic rate of L6 myotubes, and found BPA did not alter glycolysis at environmentally-relevant concentrations. Interestingly, at high concentrations of BPA (10^5 nM), there was an increase in glycolytic rate in these cells (~2-fold in basal and oligomycin treated cells). Next, we measured the effects of BPA on glucose uptake into L6 myotubes. Glucose uptake was not altered in skeletal muscle cells incubated with 1-10^4 nM of BPA. Consistent with the increased glycolytic rate, we found that L6 myotubes

exposed to 10^5 nM of BPA had increased glucose uptake (~4-fold in basal cells, ~2.5-fold in insulin treated cells), with no effects on insulin fold-increase. Furthermore, we found that L6 myotubes had increased levels of insulin-induced phosphorylation of Akt. One possible reason for the difference in insulin sensitivity is the use of 100 nM of insulin, which has been shown to stimulate maximal glucose uptake in L6 cells (Ueyama et al., 1999). Therefore, it is possible that with high concentrations of BPA there is no change in insulin-stimulated glucose uptake because 100 nM insulin already induced maximal glucose uptake (Ueyama et al., 1999). Furthermore, the phosphorylation of Akt is involved in different signaling pathways such cell survival and apoptosis, oxidative stress, and glucose uptake (Kim et al., 2001). It is thus also possible that Akt was phosphorylated at high concentration of BPA due to other signaling pathways such as the cell survival pathway due to the high concentration used. Future studies are required to look at other signaling markers in the insulin signaling pathway and downstream markers of Akt in other pathways, such as the cell survival and apoptosis pathway, in cells exposed to BPA.

Increased glucose uptake of L6 myotubes exposed to BPA brings into question how BPA exposure is linked to the development of muscle insulin resistance. Alonso-Magdalena et al. (2006) showed that mice exposed acutely to BPA (10 μg/kg) had increased plasma insulin levels and reduced glycemia, corresponding to improved insulin sensitivity. Conversely, they showed that chronic exposure to BPA resulted in more potent hyperinsulinemia and insulin resistance (Alonso-Magdalena et al., 2006). Mullainadhan et al. (2017) had shown that rats treated with 10, 100, and 400 mg/kg bw/day of BPA chronically (30 days) were insulin resistant and had reduced insulin receptor β protein levels and tyrosine phosphorylation, phosphorylation of IRS1 and Akt in the skeletal muscle. Therefore, it is possible that acute BPA exposure does not directly affect skeletal

muscle metabolism, however, long-term (chronic) exposure may result in insulin resistance in the skeletal muscle.

The increased glucose metabolism at high concentrations of acute BPA seen in our study is likely a mechanism to compensate for BPA-induced mitochondrial dysfunction. This was demonstrated in one study, where transgenic knockout mice for mitochondrial genes have increased insulin action, glucose tolerance, and enhanced glucose uptake in the skeletal muscle (Wredenberg et al., 2006). Similarly, in another study Pospisilik et al. (2007) showed deletion of the mitochondrial apoptosis-inducing factor, which is required for normal functioning of the mitochondrial respiratory chain leads to improved insulin sensitivity and glucose uptake. Interestingly, Sakurai et al. (2014) showed that 3T3-L1 cells exposed to BPA for 24 hours had increased glucose uptake and GLUT4 expression at high concentrations (10^5 nM), with no effect at lower concentrations (1 nM - 10^4 nM), which closely resembles our results. Taken together, these results emphasize that acute BPA exposure and subsequent mitochondrial dysfunction can lead to increased activity of glycolysis and phosphorylation of Akt to compensate for reduced energy production by the mitochondria.

Several studies have demonstrated that there is no specific mechanism by which BPA exerts its effects. In general, it is known that BPA is an EDC and has the ability to mimic E2 by binding to the ER. Therefore, we used the estrogen receptor antagonist ICI to determine if mitochondrial dysfunction and increased glucose metabolism in L6 myotubes at 10^5 nM is due to binding to the ER. We found that at 10^5 nM of BPA, ICI inhibited the increase in glucose uptake in L6 myotubes. Interestingly, however, ICI was unable to prevent BPA-induced mitochondrial dysfunction. Although BPA is able to exert its effects by binding to the ER, it has also been shown to exert its effects through a nonclassical pathway, such as binding the ERRγ. This concept was highlighted

in study by Alonso-Magdalena et al. (2006), that showed that in mice treated with BPA (50 μg/kg) ICI was able to reverse the increase in pancreatic insulin content, however, ICI had no effect of serum glucose levels or insulin release in mice treated acutely with BPA. Therefore, it is possible that BPA can exert its mechanism of action by the classical and non-classical ER pathways, both *in vivo* and *in vitro*.

5.2 The effects of BPA on adipose tissue adipokine expression and glucose metabolism

Due to their lipophilic nature, bisphenols have been shown to accumulate in adipose tissue and as a result may induce metabolic disruption by mimicking or blocking endogenous hormones (Wang, Xue, and Kannan, 2015). Adipose tissue plays an important role in whole-body metabolism, as it is not only a fat reservoir, but also an endocrine organ (Coelho, Oliviera, and Fernandes, 2013). Adipocytes store FA as TGs as a source of energy, and TGs are released as FA when required. Furthermore, adipocytes can release adipokines that function in an autocrine or paracrine manner. Adipocytes exposed to BPA have been shown to have increased adipogenesis, altered adipokine levels, and reduced activity of the insulin signaling pathway (De Filippis, Li and Rosen, 2018; Desai et al., 2018). Moreover, although the effects of the BPA analog, BPS, on adipocyte metabolism are even less understood, BPS has been shown to increase gene expression of adipogenic markers, and lipid accumulation in adipocytes (Ahmed and Atlas, 2016; Boucher, Ahmed and Atlas, 2016).

Whilst studies exist investigating the direct effects of bisphenols on adipocyte metabolism, seldom studies have looked at the effects of direct BPA or BPS exposure on whole adipose tissue. Whole adipose tissue culture provides substantial benefits when investigating long-term gene expression

of adipocytes in adipose tissue (Carswell, Lee, and Fried, 2015). One reason for this is that adipocyte specific gene expression has been shown to be lost when isolated from adipose tissue (Carswell, Lee, and Fried, 2015). For example, loss of adipocyte gene expression of GLUT4, and loss of insulin sensitivity, has been reported in isolated rodent adipocytes (Sivitz, DeSautel, Lee and Pessin, 1992). Therefore, in this study we investigated the effects of BPA and BPS on inflammation expression and glucose metabolism in human adipose tissue explants, since adipose tissue dysfunction plays an important role in energy and glucose homeostasis.

Adipose tissue insulin resistance is closely linked to increased inflammation (Gregor and Hotamisligil, 2011). It has been shown that 3T3-L1 adipocytes cultured with BPA (1 nM and 3 nM) have increased expression of proinflammatory markers such as *TNFA* and *IL-6*, whereas others studies have shown that BPS (0.1 µM) has been shown to reduce IL-6 expression in human adipocytes (De Filippis, Li and Rosen, 2018; Peshdary et al., 2020). The effects of BPA or BPS on expression of inflammatory markers in whole adipose tissue has not been studied. Therefore, we wanted to determine whether human adipose tissue explants exposed to BPA or BPS would have similar alterations in inflammation. Interestingly, we found reduced levels of the proinflammatory cytokines *IL-6* (1 and 10 nM), *IL-1B* (10 nM), and *TNFA* (1 nM) following 24 hour BPA exposure. These results are consistent with our positive control, dexamethasone, which is known to induce insulin resistance in adipose tissue and also reduce the expression of certain proinflammatory cytokines, such as IL-6 (Fried, Dunkin, and Greenburg, 1998), which was confirmed in the present study. Dexamethasone has been shown to reduce glucose uptake in adipose tissue following long-term (72 hour) exposure (Sarsenbayeva et al., 2019). Dexamethasone is a glucocorticoid that exerts its effects by binding to the glucocorticoid receptor. It has been shown that BPA can bind the glucocorticoid receptor in a similar fashion and with

similar binding energy to dexamethasone and cortisol (Prasanth, Divya and Sadasivan, 2010). Therefore, it is possible that in adipose tissue BPA may be exerting its effects in a similar manner to dexamethasone. As noted, these results are counterintuitive to what is expected in insulin resistance. In insulin resistance, IL-6 protein content is understood to be negatively associated with insulin-stimulated glucose disposal in adipose tissue (Nieto-Vazquez, Fernandez-Veledo, de Alvaro and Lorenzo, 2008). Although adipocytes secrete IL-6, it only accounts for approximately 10% of total tissue production, thus, other cells in the adipose tissue may contribute to increased IL-6 production (Fried, Bunkin and Greenberg, 1998). Furthermore, IL-1B mediates macrophage-induced impairments in the insulin signaling pathway, such as reduced phosphorylation of Akt expression in human adipocytes (Gao et al., 2014). Although BPA can increase inflammatory markers in adipocytes, it has also been shown to reduce proinflammatory cytokine production in macrophages (Valentino et al., 2015; Pyo, Kim, Back and Yang, 2007). Therefore, it is possible that whole adipose tissue cultured with BPA may have a different inflammatory response than isolated adipocytes due to presence of the adipose tissue stroma.

Although BPS is considered a safe alternative to BPA, recent studies have indicated that BPS exposure may have adverse health outcomes. For example, Boucher et al (2016) showed that preadipocytes treated with high concentrations of BPS (10 μM) have lipid accumulation and increased gene expression of adipogenic markers. In our study, we found no changes in expression of proinflammatory cytokines in adipose tissue exposed to BPS for 24 hours. It is possible that at environmentally-relevant concentrations, BPS does not alter levels of inflammatory markers in adipose tissue. In addition, we found that adipose tissue treated with BPA or BPS for 72 hours did not have a similar reduction in proinflammatory cytokine expression. This may be the result of an adaptive mechanism of the adipose tissue to counteract

the effects of BPA exposure. Since the half-life of BPA and BPS are approximately 6 and 7 hours, respectively, the exposure durations in our study model acute exposures that occur *in vivo*.

In this study, we found that 24 hour BPA exposure resulted in reduced levels of *FABP4* (10 nM) and *ADIPOQ* (10 nM), with no effect on *LEP* levels. The release of *FABP4* from adipocytes and macrophages is positively related to obesity and insulin resistance (Nakamura et al., 2017). The reduction of *FABP4* in our study is consistent with a study that has shown that 3T3-L1 exposed to BPA (0.1 nM-3 nM) during differentiation have reduced mRNA levels of *FABP4* (De Filippis, Li and Rosen, 2018). The adipokine, adiponectin, has been shown to be inversely related to adiposity, and increased plasma adiponectin levels are directly correlated with improved insulin sensitivity. Reduced adiponectin expression in our study corresponds to the study by Hugo et al. (2008) that showed pre-adipocytes isolated from human abdominal subcutaneous adipose tissue explants incubated for 6 hours with 0.1, 1, and 10 nM of BPA to have reduced levels of adiponectin. Furthermore, they showed that this did not occur through the estrogen receptor, as effects were not reversed by the use of an estrogen receptor antagonist (ICI 182,780) (Hugo et al., 2008). It was unclear, however, whether BPA or BPS is able to alter levels of estrogen receptors. It has previously been shown that BPA enhances ER expression in Jurkat cells, however, it is unknown whether this occurred in adipose tissue as well (Cipelli et al., 2014). Therefore, we measured levels of *ESR1* and *ESR2* to determine if BPA or BPS exposure can alter expression levels. We found no significant changes in levels of these receptors. Although BPA has been linked to enhanced ER expression (Melzer et al., 2011), it is unlikely that this occurs in human adipocytes or adipose tissue.

In this study, subcutaneous adipose tissue was used to investigate the effects of BPA and BPS, however, variations exist between the regional distribution of adipose tissue in the body. Visceral fat, for example, is linked to hyperlipidemia, insulin resistance, and T2D (Wajchenberg, Giannella-Neto, da Silva and Santos, 2002). Studies have shown heterogeneity between subcutaneous, abdominal, and gluteal adipose tissue (Wajchenberg, Giannella-Neto, da Silva and Santos, 2002). Furthermore, it has been shown that molecular mechanisms of adipocytes in these adipose tissue depots differ. For example, visceral adipose tissue has lower insulin sensitivity, transfers and releases fatty acids more extensively, produces higher levels IL-6, and lower levels of leptin and adiponectin, in comparison to subcutaneous adipose tissue (Wajchenberg, Giannella-Neto, da Silva and Santos, 2002). It is possible that BPA and BPS can accumulate in visceral adipose tissue and exert a different effect in comparison to subcutaneous adipose tissue. In one study, the effects of environmentally-relevant concentrations of BPA on subcutaneous and visceral breast adipocytes were investigated (Hugo et al., 2008). Interestingly, they showed that BPA-induced adiponectin decrease in these adipocytes was reversed by an estrogen receptor antagonist in visceral, and not subcutaneous adipose tissue (Hugo et al., 2008). Therefore, this highlights the possible differences in mechanism of action of bisphenols in different adipose depots, and future studies should investigate these possible differences.

Impaired glucose metabolism by the adipose tissue has been associated with the development of insulin resistance, therefore, in this study we incubated adipose tissue with BPA for 24 hours to determine whether glucose metabolism would be altered. We showed that adipocytes isolated from adipose tissue exposed for 24 hours with low concentrations of BPA had a reduction in insulin-stimulated glucose uptake, with no effects on basal glucose uptake. This coincides with previous studies that have shown that adipocytes exposed to BPA have reduced insulin-stimulated glucose

uptake (De Filippis, Li and Rosen, 2018). Interestingly, we found that 24 hour BPA exposure did not alter total levels or phosphorylation of Akt, protein levels of GLUT4, or gene expression of *AKT*, *IRS1*, *GLUT1*, and *GLUT4*. Other diabetogenic drugs, such as the immunosuppressive agents cyclosporin A and tacrolimus, can also inhibit glucose uptake, without altering activation/phosphorylation of the insulin signaling proteins IRS1/2, AS160, GLUT1, and GLUT4, but by altering the translocation of GLUT4 to the membrane (Pereira et al., 2014). Due to the difficulty of isolating sufficient adipocytes for isolation of the plasma membrane to measure GLUT4 (Pereira et al., 2014), future studies should determine an appropriate protocol to investigate the effects of BPA on GLUT4 transport machinery and overall translocation to the plasma membrane. Furthermore, studies on the effect of BPA on adipocyte and adipose tissue glucose metabolism often show conflicting and difficult to interpret results due to differences in incubation time and the wide range of concentrations used. As mentioned earlier, in one study, 24 hour BPA exposure of 3T3-L1 adipocytes lead to increased glucose uptake and GLUT4 expression in adipocytes treated with 10^5 nM of BPA, with no effect at concentrations between 1 nM - 10^4 nM lower concentrations (Sakurai et al., 2004). Contrastingly, another study using human adipocytes showed incubation with 1 and 100 nM of BPA for 8 hours had no effect on glucose uptake, whereas 24 and 48 hours having reduced glucose uptake, with the latter having the greatest reduction (Valentino et al., 2013). This highlights the importance of exposure duration and concentration with BPA or BPS, and it is apparent that chronic low dose exposure may have important implications on metabolism.

Taken together, this study provides first evidence on the effects of BPA and BPS on inflammatory markers and glucose uptake in human subcutaneous adipose tissue. Future studies are required to

understand the mechanism by which glucose uptake is reduced in adipose tissue, and whether the effects of BPA on adipose tissue can affect inter-organ cross-talk.

5.3 Communication between the adipose tissue and skeletal muscle: a possible cause for BPA-induced metabolic dysfunction in the skeletal muscle?

Since *in vivo* models have shown impaired glucose metabolism in the skeletal muscle following BPA exposure, we investigated the effects of direct BPA exposure on skeletal muscle cells. Specifically, we wanted to determine whether impaired muscle glucose metabolism was due to direct interaction between muscle cells and BPA exposure. Interestingly, we found no effect at environmentally-relevant concentrations of BPA, which may be due to the absence of inter-organ crosstalk. For example, adipose tissue secretes lipids and adipokines that have an importance in skeletal muscle metabolism. As mentioned previously, in the study by Alonso-Magdalena et al. (2006) it was shown that mice exposed acutely to a low dose of BPA (10 μg/kg) improved insulin sensitivity (increased insulin, and reduced glycemia). In our study, we show that at a low concentration of BPA there are no effects on L6 glucose metabolism. Interestingly, adipose tissue incubated for 24 hours with BPA have reduced levels of proinflammatory cytokine expression, which may contribute to the overall improvement in glucose metabolism of the skeletal muscle. Furthermore, although we show that 24 hour exposure leads to reduced glucose uptake in adipose tissue, the skeletal muscle accounts for a great majority (~80%) of postprandial glucose disposal. Therefore, despite reduced glucose uptake in the adipose tissue that we see in our study, improved insulin sensitivity of the skeletal muscle, as seen in the study by Alonso-Magdalena et al. (2006), may occur due to the fact that most insulin-stimulated glucose disposal occurs in the muscle.

Therefore, future studies should focus on the effects of acute BPA exposure on crosstalk between the adipose tissue and the skeletal muscle to determine if adipokines secreted from adipose tissue explain improved skeletal muscle metabolism in the mice (Alonso-Magdalena et al., 2006).

Adipose tissue dysfunction can alter the metabolism of peripheral tissue through increased release of FFA (ie. through lipolysis) and increased ectopic lipid deposition in organs such as the skeletal muscle (Gancheva, Jelenik, Álvarez-Hernández and Roden, 2018). It is known that under normal conditions, lipolysis is inhibited by insulin (Chakrabarti et al., 2013). Although it is currently unclear whether BPA alters lipolysis in adipose tissue, *in vitro* studies have shown that BPA induces dysfunction of pancreatic islet cells, including reduced insulin secretion and increased apoptosis (Carchia et al., 2015). It is possible that BPA exposure alters insulin release and, thus, inhibition of lipolysis. Increased lipolysis and release of FFA may lead to increased lipid accumulation in the skeletal muscle. In lean individuals the skeletal muscle can store about 0.5% of lipid droplet volume density, however, this increases to almost 1.5% in individuals with obesity (Gancheva, Jelenik, Álvarez-Hernández and Roden, 2018). Furthermore, skeletal muscle lipid accumulation is negatively associated with insulin sensitivity (Gancheva, Jelenik, Álvarez-Hernández and Roden, 2018). This highlights the possibility of impaired adipose tissue metabolism and release of adipokines and lipids due to BPA exposure may be able to induce insulin resistance in the muscle. Future studies should investigate adipose-to-skeletal muscle crosstalk in order to better understand inter-organ communication.

5.4 Limitations and future directions

In this study, we investigated the direct effects of acute BPA exposure on skeletal muscle metabolism. In reality, it is probable that BPA exposure is chronic since we are constantly exposed

to common sources of BPA such as plastics, receipts, and epoxy lined products. Furthermore, exposure to BPA can occur through a variety of routes such as oral consumption, transdermal absorption, and respiration. The most common forms of BPA exposure are oral and transdermal exposure. The Scientific Committee on Food has reported that the maximum amount of BPA in blood following oral ingestion is 2-8%, compared to 27-51% following intraperitoneal injection, or 65-76% following subcutaneous injection. Therefore, the amount of free BPA that is released into the circulation following exposure is relatively low. This highlights one limitation of this study, since we use free BPA *in vitro*, whereas *in vivo* there are low levels of free BPA. Future studies should investigate the adverse effects of BPA metabolites, such as BPA-G, on muscle metabolism *in vitro*.

Another limitation of this study is the use of L6 cells, which are cells isolated from the thigh of newborn rats (Cho and Doles, 2017). Although the use of L6 cells is beneficial for investigating metabolism and nutritional cues, it may not completely recapitulate what occurs in human primary muscle cells and in the muscle tissue. For example, it has been shown *in vitro* that human primary muscle cells having higher levels of GLUT3, citrate synthase, and myosin heavy chain associated with type 1 fibers, which suggest that human primary muscle cells better represent adult oxidative fibers compared to L6 cells (Cho and Doles, 2017). In addition, the nature of the *in vitro* myotubes may not exactly represent mature intact muscle that are *in vivo*. The lack of the physiological microenvironment, such as blood flow and the cell-extracellular matrix is another limitation with this model. Furthermore, the lack of flow across the skeletal muscle may allow for the accumulation of factors used *in vitro* (ie. bisphenols), which may lead to levels that are supraphysiological if treatment is changed repeatedly (ie. every day during differentiation) (Cho and Doles, 2017).

Interpatient variability is another limiting factor of our study. Variability in adipose tissue composition between individuals can be due to differences in gender, environmental factors, and BMI. Gender has been shown to dictate fat storage, hormone secretion, and the brain's response to hormones (Shi and Clegg, 2009). Certain studies have shown that women have differential expressions of the ER in comparison to men. For example, it has been shown that estradiol signaling mediates sex differences in visceral adipose tissue, as males have lower levels of ERα than females, which corresponds to higher visceral adiposity in males (Tao et al., 2018). Therefore, since BPA and BPS can exert their effects through ER signaling pathways, there may be gender differences in adipose tissue exposure to these compounds. Due to limitation in replicates, there were not enough subjects to make conclusions about the correlation between the sex of the subject and the effects of BPA. Although one of the subject exclusion criteria for obtaining adipose tissue was having T2D, some patients may have had better insulin sensitivity compared to others due to the wide range of BMI used in this study. Obesity is considered one of the greatest risk factors for the development of insulin resistance (Blüher, 2016). Furthermore, studies have shown that adipose tissue from individuals with obesity have reduced insulin sensitivity compared to lean individuals. This was demonstrated by Stolic et al. (2002), who showed that adipose tissue from lean subjects had approximately a two-fold increase in transport of glucose in response to insulin, however, glucose uptake in adipose tissue of obese or overweight subjects were less responsive to insulin. Therefore, BPA and BPS on adipose tissue may have a different effect depending on the BMI and insulin resistance of the subject. In addition, due to the lipophilic nature of BPA and BPS, higher levels can accumulate in the adipose tissue of individuals with obesity compared to lean individuals, which may in turn alter the overall effect we measured *in vitro*. Specifically, if BPA or BPS is accumulated in the adipose tissue, the overall circulating levels are lower, than in an

individual with less adipose tissue (Lee, Jacobs, Lind and Lind, 2020). Therefore, adipose tissue from individuals with higher BMI may have higher basal levels of BPA, and thus metabolic dysfunction, than individuals who have lower BMI. Furthermore, certain studies have shown that individuals with obesity are protected from the adverse effects of BPA due to accumulation in the adipose tissue, however, the adverse effects of bisphenols are increased following weight loss due to release of BPA from the adipose tissue (Lee, Jacobs, Lind and Lind, 2020). Thus, adipose tissue derived from individuals that have recently had weight loss may also exhibit a different phenotype when compared to individuals of the same weight without recent weight loss. Due to low numbers of replicates in our study with adipose tissue, correlation analysis was not performed. Future studies should investigate the effects of BPA on adipose tissue, taking into account differences in metabolic parameters, such as BMI and glycated hemoglobin levels.

6. Conclusions

In this study, we investigated the effects of BPA and BPS on muscle and adipose tissue metabolism. We show that at environmentally-relevant concentrations, BPA does not alter mitochondrial function or glucose metabolism of L6 myotubes. Interestingly, high concentrations of BPA (10^5 nM) resulted in reduced mitochondrial function, and increased glucose metabolism, which was likely to compensate for reduced energy production by the mitochondria. Adipose tissue treated with BPA for 24 hours had reduced expression of certain inflammatory markers and adipokines, however, BPS treatment did not alter inflammatory marker or adipokine expression in adipose tissue. Furthermore, we found that adipose tissue treated with BPA for 24 hours had reduced insulin-stimulated glucose uptake, without altering gene expression or protein levels of key markers in the insulin signaling pathway. Taken together, our study demonstrates that BPA at environmentally-relevant levels can alter adipose tissue glucose metabolism and adipokine expression, however, has no effect on skeletal muscle glucose metabolism or mitochondria. This calls for re-examination of current guidelines on the daily tolerant doses of BPA, and improved regulation on the use of BPA in consumer products.

7. References

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8. Contributions

The investigation on the effects of acute BPA exposure on L6 muscle cells was done at the University of Ottawa under the supervision of Dr. Céline Aguer. Seahorse XF experiments, radioactive glucose uptake, and metabolic activity experiments were done, in part, by Lucia Chehade.

The investigation on the effects of BPA and BPS exposure on adipose tissue was done at Uppsala University, under the supervision of Dr. Maria Pereira.

9. Appendix

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The effects of acute BPA exposure on skeletal muscle mitochondrial function and glucose metabolism

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ABSTRACT

Bisphenol A (BPA) is an environmental pollutant that has been associated with adverse health effects including skeletal muscle insulin resistance, a major contributor to the pathogenesis of type 2 diabetes (T2D). Early mitochondrial dysfunction and oxidative stress are linked to impaired glucose metabolism in skeletal muscle. In this study, we investigated the effects of BPA on skeletal muscle mitochondrial function and insulin sensitivity. L6 myotubes were treated with BPA (1 nM–10^5 nM) during the last 24 h of differentiation. Following exposure to 10^5 nM of BPA, resting and maximal oxygen consumption rates were decreased, whereas mitochondrial proton leak was increased. Overall metabolic activity, measured by redox ability, was decreased in L6 myotubes exposed to 10^5 nM of BPA. At this concentration, insulin-stimulated glucose uptake was increased, which corresponded to an increased phosphorylation of the insulin signaling protein Akt, and increased glycolysis measured by extracellular acidification rate (ECAR). Acute BPA exposure did not alter levels of oxidative stress markers in muscle cells, but significantly increased mitochondrial proton leak, which is known to be involved in decreased ROS production. The effects of BPA on glucose uptake, but not mitochondrial function, were reversed by the use of an estrogen receptor antagonist. These results suggest that acute exposure of L6 myotubes at only high concentrations of BPA alters glucose metabolism, which is likely a compensatory response to reduced mitochondrial energy production capacity.

1. Introduction

The development of type 2 diabetes (T2D) is often associated with poor nutritional habits, such as increased carbohydrate consumption. Although it is implicated in the development of T2D, the start of the diabetes epidemic does not directly correlate to the increased sugar consumption (Pizzorno, 2016). Exposure to pollutants has been linked to the development of T2D, as increased body load of pollutants is associated with increased odds ratio for the development of T2D (Pizzorno, 2016; Duan et al., 2018). For example, increased urine content and body load of pollutants, such as bisphenols, have been positively associated with the development of this chronic disease (Pizzorno, 2016; Duan et al., 2018).

Bisphenol A (BPA) is a synthetic monomer used in the production of polycarbonate plastics such as food containers and bottles (Duan et al., 2018). BPA can migrate from polycarbonate plastics into food and beverages, which has proven to be a concern since BPA has been linked to adverse health effects (e.g. metabolic diseases) (Ben-Jonathan and Steinmetz, 1998; Lin et al., 2013). BPA is an endocrine disruptor, with the ability to bind to estrogen receptors (ERs), and act as either an agonist or antagonist (Gould et al., 1998). Mice exposed to a single dose (10 μg/kg) of either 17β-estradiol (E2) or BPA had significantly reduced glycemia that corresponded to increased plasma insulin levels (Alonso-Magdalena et al., 2006). Furthermore, the same group demonstrated that chronic exposure to low doses of BPA (10 μg/kg/day for 4 days) in mice results in hyperinsulinemia and insulin resistance (Alonso-Magdalena et al., 2008). Although it is clear that BPA exposure is associated with altered energy metabolism, the mechanism by which this pollutant leads to changes in glucose metabolism is currently unclear.

Environmentally-relevant concentrations of BPA have been shown

to be in the low nanomolar range, as described in the review by Welshons et al. (2006). For example, BPA has been detected at concentrations between 1 and 20 nM in serum, breast milk, and even umbilical cords at birth (Takeuchi and Tsutsumi, 2002; Sun et al., 2004; Todaka and Mori, 2002). Low dose BPA exposure during development has been shown to cause insulin hypersecretion in rat offsprings (Manukyan et al., 2019). Some studies have investigated the effects of high doses of BPA (micromolar) since BPA is known to follow a non-monotonic dose response, in which the effects at low concentrations may not be predicted at high concentrations (Vandenberg et al., 2012). One study looking at the effect of BPA on adipocytes found that BPA had certain effects such as accelerated adipogenesis at concentrations above 80 μM, whereas another study showed that BPA had an effect on adipose tissue glucose uptake from 100 μM (Masuno et al., 2005, Sakurai, Kawazuma et al. 2004).

In hepatocytes, BPA exposure has been linked to reduced levels of antioxidant enzymes, leaving cells unprotected from BPA-induced oxidative stress, which results in subsequent mitochondrial dysfunction (Moon et al., 2012). While hepatocytes are known to be more susceptible to the adverse effects of xenobiotics at low concentrations, studies have shown similar mitochondrial toxicity in skeletal muscle with other types of xenobiotics (Dott et al., 2018). Hence, the observed adverse effects of BPA in hepatocytes may be similarly reflected in skeletal muscle.

Skeletal muscle accounts for 80% of postprandial glucose disposal; therefore, insulin resistance in this tissue is a major contributor to the pathogenesis of T2D (DeFronzo and Tripathy, 2009). Specifically, impaired muscle glucose metabolism and mitochondrial function are linked to the development of insulin resistance (Kelley et al., 1996; Kelley et al., 2002). Although mitochondrial dysfunction is not necessarily a precursor to insulin resistance, it is evident that it can ultimately contribute to its development and progression (Kim et al., 2008).

BPA exposure is often associated with the development of T2D; however, its mechanism of action has not been completely characterized. Since BPA has been shown to be associated with mitochondrial dysfunction in certain cell types, it is possible that BPA also affects oxidative metabolism in skeletal muscle. Taken together, this warrants further investigation of the direct effects of BPA on skeletal muscle mitochondrial function and insulin sensitivity. The aim of our study was to better understand the link between BPA exposure and impaired muscle glucose metabolism.

2. Materials and methods

2.1. L6 muscle cell culture and treatment

Rat L6 myoblasts, kindly provided by Dr. Amira Klip, were grown to 70% confluence in α-minimal essential medium (α-MEM; Wisent, Canada) supplemented with 10% fetal bovine serum (FBS; Wisent, Canada) and 1X AA (Antibiotics - Antimycotics; Wisent, Canada). Cells were differentiated over a period of 7 days into myotubes in α-MEM with 2% FBS (supplemented with 1X AA). Myotubes were exposed to different concentrations of BPA (1, 10, 10^2, 10^3, 10^4, 10^5 nM) or the vehicle (0 nM BPA; 0.1% DMSO (Sigma-Aldrich, Canada)) for the last 24 h of differentiation.

2.2. Cell viability

L6 myoblasts were plated on a 96-well plate at 10^4 cells per well. After 7 days of differentiation and BPA treatments, cell viability was assayed spectrophotometrically using lactate dehydrogenase (LDH) Assay Kit (ab65393, Abcam, USA) according to the manufacturer's instructions. L6 myotubes were briefly exposed to LDH reaction mix for 30 min and absorbance was measured at 450 and 650 nm (reference wavelength). Cell viability was calculated according to the equation [(test sample absorbance - low control absorbance)/(high control absorbance - low control absorbance)]. High control cells were lysed with lysis buffer, according to manufacturer's instructions. Low control cells were untreated L6 myotubes. Cell cytotoxicity was normalized to 0 nM BPA (vehicle).

2.3. Mitochondrial metabolic activity

L6 myoblasts were plated on a 96-well plate at 2 x 10^4 cells per well. After 7 days of differentiation and BPA treatments, mitochondrial metabolic activity was assayed spectrophotometrically using PrestoBlue® reagent (Life technologies, Canada) according to manufacturer's instructions. Myotubes were incubated for 30 min in 1X PrestoBlue® reagent and absorbance was measured at 570 and 600 nm (reference wavelength). The PrestoBlue® assay is often used for determining cell cytotoxicity, however, due to the nature of its mechanism, it can also be used for investigating metabolic activity. Specifically, this resazurin-based metabolic assay uses the reducing ability of cells to modify the PrestoBlue reagent (convert resazurin to resorufin), which is quantitatively measured by color change. Resazurin is effectively reduced within mitochondria, allowing to test for mitochondrial metabolic activity as well (Sonnaert et al., 2015).

2.4. Oxygen consumption and extracellular acidification rate measurements

L6 myoblasts were plated on a XF96-well plate (8 x 10^3 cells per well) or a XF24-well plate (3 x 10^4 cells per well) (Seahorse Bioscience Inc., USA). After 7 days of differentiation and treatments, myotubes were washed and incubated for 45 min at 37°C at ambient CO_2 in HCO_3-free assay Dulbecco's Modified Eagle Medium (DMEM) containing 4 mM glutamine, 1 mM sodium pyruvate and 5 mM dextrose (all from Sigma-Aldrich, Canada) (Seahorse assay medium; pH 7.4). Oxygen consumption rates (OCR) and extracellular acidification rates (ECAR; as a marker of glycolysis) were measured using the Seahorse extracellular flux analyzer XF-96 or XF-24 at 37°C (Agilent, USA). Rates were obtained in presence of oligomycin (3 μM), carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP; 2 μM) and antimycin A (4 μM) (all from Sigma-Aldrich, Canada), corresponding to state 4 respiration, maximal respiration (state 3 non-coupled respiration), and non-mitochondrial OCR, respectively. Following measurements, cells were lysed using 0.05 M NaOH and protein content was assayed using the Bradford protein assay (Biorad, Canada). Mitochondrial OCR and ECAR are expressed per μg of total cellular proteins. Mitochondrial respiration was corrected for by subtracting all OCR rates by OCR following antimycin treatment (non-mitochondrial OCR). Since respiratory CO_2 contributes to the extracellular acidification, ECAR values were corrected for CO_2 production as described by Mookerjee et al. (2015). It is important to note that maximal ECAR (in the presence of oligomycin) is only an approximation of maximal glycolysis, since it may be limited by ATP demand.

2.5. Glucose uptake

L6 myoblasts were plated at 10^5 cells per well, differentiated, and exposed to BPA or vehicle for 24 h in 2% FBS α-MEM. Cells were then starved for 3 h using serum-free α-MEM. L6 glucose uptake was measured as described in Klip et al. (1986). Half of the cells were treated with insulin (100 nM) for 20 min at 37 °C. Cells were washed 3 times and then incubated in HEPES-Buffered Saline with 10 μM 2-deoxyglucose and 0.5 μCi/ml $^3H^3$ 2-deoxyglucose. Cytochalasin B (10 μM) was used to determine non-specific glucose uptake. Myotubes were lysed in 0.5 ml of 0.05 M NaOH and 0.4 ml was analyzed by Tri-Carb2910TR scintillation counter (PerkinElmer, Canada). Protein content was measured using Bradford protein assay in the remaining 0.1 ml of the cell lysate. Glucose transport activity is expressed as pmol of 2-deoxy-glucose taken up/min/μg of total protein.

2.6. Inhibition of estrogen receptor

To block the estrogen receptor, L6 myotubes were pretreated with the antiestrogen fulvestrant (ICI 182, 780) (10 nM) (Sigma-Aldrich, Canada) for 30 min, followed by co-treatment with BPA (10^5 nM) for the last 24 h of differentiation. Glucose uptake, OCR, and ECAR, were measured as previously described.

2.7. Western blots

L6 myoblasts were plated at 3 x 10^5 cells on a 6-well plate, differentiated, and exposed to BPA or vehicle for 24 h. L6 myotubes were lysed in lysis buffer (20 mM Tris-HCl, 50 nM NaCl, 250 mM sucrose, 1% 100X Triton, 50 mM NaF, 5 mM NaP). Monoclonal anti-glutathione peroxidase 1 (ab108427), monoclonal anti-glutathione peroxidase 4 (ab125066), polyclonal anti-glutaredoxin 2 (ab191292), monoclonal anti-Akt1 (phospho serine S473) (ab81283), polyclonal anti-pan-Akt antibody (ab8805), MitoProfile® Total OXPHOS Rodent WB Antibody Cocktail (ab110413) (all from Abcam, Canada), monoclonal anti-catalase (CST-D5N7V, Cell Signaling Technology, Canada), polyclonal anti-α-tubulin (CST-2144, Cell Signaling Technology, Canada), and polyclonal superoxide dismutase (SOD2) (sc-30080, Santa Cruz, USA) were used as primary antibodies at a dilution of 1:1000, except for the anti-Akt1 (phospho serine 473) antibody, which was diluted 1:500. The secondary antibodies used were anti-mouse (sc-516102) and anti-rabbit (sc-2357) antibodies coupled to horseradish peroxidase (Santa Cruz, USA), diluted 1:5000. Proteins were visualized using SuperSignal West Pico Western Blot Kit (34580, Thermo Scientific, USA) or Clarity Western ECL Substrate (170–5061, Biorad, USA) and imaged using ChemiDoc™ Imager and VisionWorks LS (UVP, USA). Expression of proteins was quantified by densitometry analysis using ImageJ program (National Institutes of Health, USA).

2.8. Statistical analysis

Data are presented as means ± SEM. Unpaired 2-sided *t* tests, one-way analysis of variance (ANOVA), and two-way ANOVA with a Fisher PLSD *post hoc* test and Tukey's multiple comparisons test were used to assess statistical differences with Statview 5.0 Software (SAS Institute, USA) and GraphPad Prism version 6.0e (La Jolla, USA). Values with $p < 0.05$ were considered significant.

3. Results

3.1. Acute BPA exposure does not alter cell viability of L6 myotubes

The effect of BPA exposure on L6 myotubes viability was first evaluated. Release of LDH, a marker of cell cytotoxicity, was spectrophotometrically measured in L6 myotubes exposed to BPA (1 nM – 10^5 nM) for 24 h. As shown in Fig. 1, BPA exposure from 1 nM – 10^5 nM for 24 h did not significantly affect L6 myotubes viability ($p > 0.05$).

3.2. Exposure to high concentrations of BPA alters mitochondrial function and metabolic activity of L6 myotubes

To determine whether BPA exposure alters mitochondrial function in skeletal muscle cells, L6 myotubes were exposed to different concentrations of BPA for 24 h and OCR was measured in basal conditions and following oligomycin and FCCP treatment (Fig. 2 and Fig. S1A). BPA exposure between 1 nM–10^4 nM did not significantly alter basal respiration in the presence of glucose, pyruvate, and glutamine as substrates; however, basal respiration was significantly decreased at 10^5 nM of BPA (Fig. 2A) ($p < 0.05$). L6 myotubes exposed to 10^5 nM of BPA also showed an increased mitochondrial proton leak whereas maximal respiration was reduced compared to the control group (Fig. 2A). The percentage of basal OCR due to proton leak was

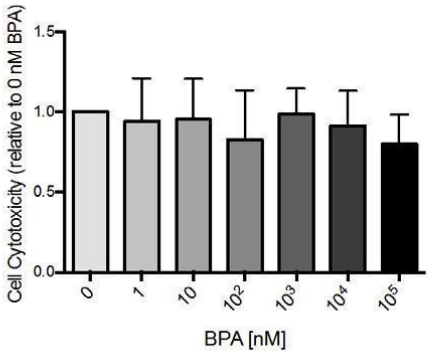

Fig. 1. The effects of 24-h BPA exposure on L6 cells viability. Cell cytotoxicity measured by lactate dehydrogenase (LDH) release from L6 myotubes following BPA (1 nM–10^5 nM) relative to the vehicle control (0 nM BPA; 0.1% DMSO). Cell cytotoxicity describes the ratio of LDH released between the test sample (BPA-treated cells) and a positive control (lysed cells). n = 3, each independent experiment was done in triplicate. Data represent mean ± SEM. *$p < 0.05$ (one-way ANOVA).

increased in L6 myotubes exposed to 10^5 nM of BPA (Fig. 2B) ($p < 0.05$). Mitochondrial metabolic activity (reducing capacity of mitochondria) was also lower in L6 myotubes exposed to 10^5 nM of BPA (Fig. 2C) ($p < 0.05$). To determine if the decreased mitochondrial function with high BPA exposure was the result of altered levels of ATP synthase, we measured the protein level of ATP synthase. ATP synthase (ATP5a) levels were not significantly altered by BPA treatment (Fig. 2D).

3.3. Exposure to high concentrations of BPA increases glucose metabolism in L6 myotubes

Extracellular acidification rate was measured as an indicator of glycolytic function in the resting state and by blocking mitochondrial ATP-synthase by oligomycin as an indirect measurement of maximal ATP production through anaerobic glycolysis (Fig. 3 and Fig. S1C). Exposure to BPA between 1 nM – 10^4 nM did not alter glycolytic rate in L6 myotubes (Fig. 3A) ($p > 0.05$); however, exposure to 10^5 nM of BPA significantly increased resting and maximal glycolysis in L6 myotubes (Fig. 3A) ($p < 0.05$). Next, basal and insulin-stimulated glucose uptake was measured in L6 myotubes exposed to BPA. Cells exposed to 10^5 nM of BPA had increased uptake of 2-deoxyglucose under both basal and insulin-stimulated conditions ($p < 0.05$) (Fig. 3B), with no effect on insulin sensitivity (measured as the fold-increase in 2-deoxyglucose uptake in response to insulin) (Fig. 3C). To investigate the effects of BPA exposure on the insulin-signaling pathway, phosphorylation of the serine/threonine kinase, Akt, was measured by Western blot (Fig. 3D). Insulin-stimulated L6 myotubes exposed to 10^5 nM of BPA also showed significantly increased phosphorylation of Akt ($p < 0.05$) (Fig. 3D).

3.4. The effects of ER antagonist ICI on glucose uptake and mitochondrial function in L6 myotubes exposed to BPA

To explore the mechanism by which high concentrations of BPA exert altered glucose metabolism and mitochondrial function, we used the specific ER antagonist ICI before measuring glucose uptake, mitochondrial function and glycolytic rates (Fig. 4 and Figs. S1B and D). Co-incubation of BPA (10^5 nM) and ICI (10 nM) reversed BPA-induced alteration of glucose uptake in both the absence and presence of insulin ($p < 0.05$) (Fig. 4A), however, BPA-induced alterations of OCR and

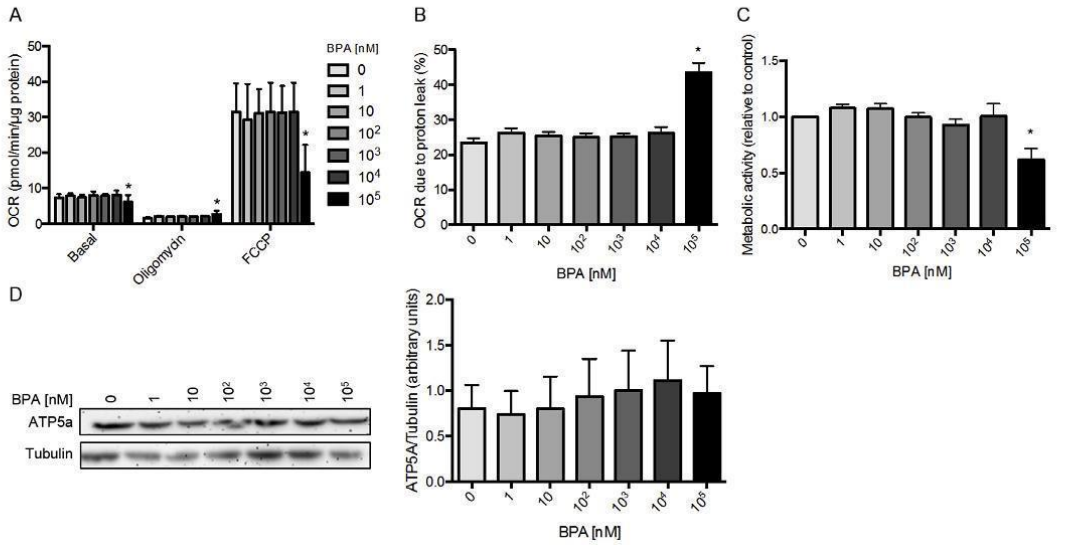

Fig. 2. The effects of 24-h BPA exposure on mitochondrial function and metabolic activity in L6 myotubes. L6 myotubes were exposed to the vehicle (DMSO) or different concentrations of BPA (1 nM − 10^5 nM) for 24 h. (A) OCR was first measured followed by treatments with: oligomycin (3 µM) to assay for proton leak, and FCCP (2 µM) to assay maximal OCR. n = 6, each independent experiment was done in 4–6 replicates. (B) Percent of basal OCR due to proton leak (C) Metabolic activity measured by PrestoBlue® assay of L6 myotubes exposed to BPA or vehicle (DMSO) for 24 h. n = 3, each independent experiment was done in 4–6 replicates. (D) Protein levels of ATP5a (ATP synthase) in L6 myotubes treated with BPA or vehicle control (DMSO) for 24 h (n = 3 independent experiments). Left panel: representative Western blot of ATP5a. Right panel: quantification of ATP5a by density analysis. α-Tubulin was used as a loading control. (A–D) Data represent mean ± SEM. *p < 0.05 compared to vehicle (one-way ANOVA).

ECAR were not reversed following co-incubation with ICI (Fig. 4B–D).

3.5. Effects of BPA exposure on oxidative stress markers in L6 myotubes

To determine the effects of 24-h BPA exposure on oxidative stress in L6 myotubes, the levels of glutathione peroxidase 1 (GPx1), GPx4, glutaredoxin 2 (Grx2), superoxide dismutase 2 (SOD2) and catalase were measured. There was no significant change in the expression of GPx1, GPx4, Grx2, catalase, and SOD2, however, there was an increased trend for SOD2 (Fig. 5A–F).

4. Discussion

Over the last decade, emerging evidence has been suggesting a strong association between BPA exposure and adverse health outcomes, including insulin resistance and T2D. In this study, we investigated the effects of acute BPA treatment on muscle energy metabolism, since skeletal muscle insulin resistance is a major contributor to the development of T2D (DeFronzo and Tripathy, 2009).

Muscle mitochondrial dysfunction has been linked to the development of insulin resistance and T2D (Mogensen et al., 2007). Although studies have shown a link between BPA exposure and the development of insulin resistance (Alonso-Magdalena et al., 2006), it was unknown whether acute BPA exposure could alter skeletal muscle mitochondrial function. Therefore, we set out this investigation and first showed that acute exposure of muscle cells to environmentally-relevant concentrations of BPA does not alter L6 myotube mitochondrial function. Interestingly, however, L6 myotubes acutely exposed to high concentrations of BPA (10^5 nM) have reduced basal and maximal mitochondrial OCR, which was confirmed by reduced metabolic activity using the PrestoBlue® assay. Although basal and maximal respiration have been shown to be reduced in patients with T2D, whereas proton leak has been shown to be increased (Phielix et al., 2008; Aguer et al., 2013), it is unlikely that skeletal muscle exposure to BPA at environmentally-relevant doses are linked to these metabolic changes. Downregulation of ATP synthase activity and levels is a common feature in obesity and T2D (Formentini et al., 2017). ATP5a level was unaltered by BPA treatment, demonstrating that reduced respiration was not due to reduced protein level of ATP synthase. Furthermore, ATP5a is often used as a mitochondrial marker (Choi et al., 2017); therefore, altered respiration was probably not due to changes in mitochondrial levels. Proteome analysis has shown reduced protein content and phosphorylation of ATP synthase protein content in individuals with T2D (Hojlund et al., 2003). In addition, there is downregulation of genes involved in oxidative phosphorylation in patients with T2D or who are at high risk for the development of T2D (Patti et al., 2003). It is likely that early mitochondrial changes due to acute BPA do not involve alteration in ATPase levels; however, this may not apply to chronic BPA exposure. These results provide some insight on the link between acute BPA exposure and skeletal muscle mitochondrial function; in which we find that there appears to be no effects at environmentally-relevant doses.

Energy production by skeletal muscle is heavily reliant on the mitochondria. It has been shown that the severity of skeletal muscle insulin resistance is related to the extent of mitochondrial dysfunction and oxidative stress (Kruszynska et al., 1998). Therefore, BPA-induced metabolic perturbations of the skeletal muscle increase the risk for the development of muscle insulin resistance. Consequently, this prompted the investigation of the effects of BPA on skeletal muscle glucose metabolism. We first estimated the glycolytic rate of L6 myotubes exposed acutely to BPA by measuring ECAR. We found that there was no effect on resting or maximal glycolytic rate between 1 nM - 10^4 nM of BPA; however, at our highest concentration of 10^5 nM, there was increased resting and maximal glycolysis. Next, we looked at glucose uptake in L6 myotubes exposed to BPA. We found that myotube exposure at concentrations of BPA between 1 - 10^3 nM had no effect on skeletal muscle

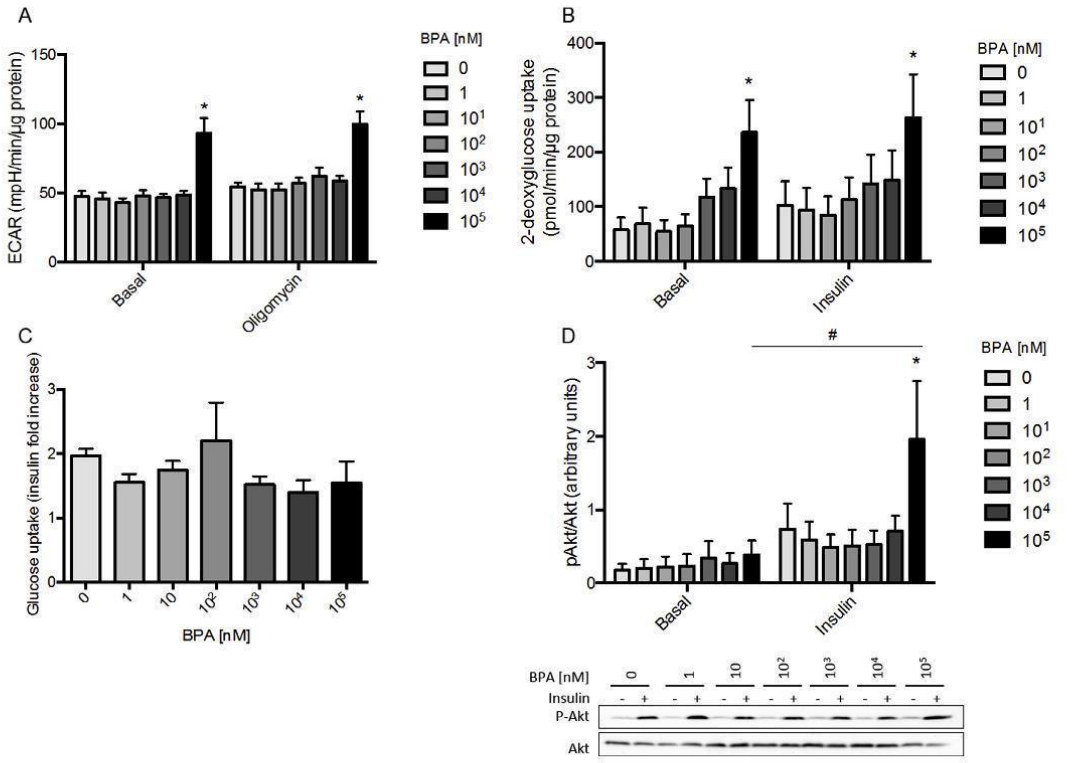

Fig. 3. The effects of 24-h BPA treatment on glucose metabolism in L6 myotubes. (A) Resting and maximal ECAR of L6 myotubes exposed for 24 h to BPA (1 nM – 10^5 nM) or vehicle (DMSO). n = 6, each independent experiment was done in 4–6 replicates (one-way ANOVA). (B) Uptake of 2-deoxyglucose and (C) fold-increase in glucose uptake in response to insulin treatment measured in L6 myotubes exposed to BPA or vehicle (DMSO). L6 myotubes were exposed to insulin (100 nM) for 20 min n = 5, each independent experiment was done in triplicate.* for $p < 0.05$ (two-way ANOVA). (D) Ratio of phospho-Akt and total Akt in L6 myotubes exposed for 24 h to BPA or vehicle (DMSO). L6 myotubes were subsequently exposed to insulin (100 nM) for 20 min. Bottom panel: representative Western blot of phospho-Akt and Akt. Top panel: quantification of phospho-Akt/Akt by density analysis. n = 3 independent experiments.*$p < 0.05$ compared to all other BPA concentrations in the same condition. # for main effect of insulin (two-way ANOVA). Data represent mean ± SEM.

glucose uptake, whereas exposure at 10^4 nM and 10^5 nM of BPA had significantly increased glucose uptake, with no effect on insulin sensitivity. Interestingly, however, myotubes exposed to 10^5 nM of BPA had increased insulin-induced phosphorylation of the insulin signaling protein, Akt. Improved insulin sensitivity and glucose uptake in skeletal muscle cells exposed to high BPA concentrations raises the question as to how BPA exposure has been linked to insulin resistance and the development of T2D. Acute BPA treatment has been shown to rapidly increase plasma insulin and reduce circulating glucose in mice, suggesting increased peripheral insulin sensitivity (Alonso-Magdalena et al., 2006). However, longer (chronic) BPA treatment resulted in increased β-cell insulin content, hyperinsulinemia and insulin resistance (Alonso-Magdalena et al., 2006). Thus, it is likely that acute myotube exposure to environmental relevant concentrations of BPA is not directly related to the changes in glucose metabolism that have been shown in in vivo models. It is possible, however, that longer BPA incubations may provide insight to the effects exhibited in mice following chronic exposure. In our study, we show that L6 myotubes treated with 10^5 nM BPA acutely, have improved glucose metabolism, which is likely a mechanism to compensate for BPA-induced mitochondrial dysfunction. This corresponds to a study by Wredenberg et al. (2006). that demonstrated that transgenic knockout mice for mitochondrial genome genes had increased insulin action, glucose tolerance, and enhanced basal and insulin stimulated 2-deoxyglucose uptake in isolated muscles, rather than insulin resistance. Additionally, acute deletion of mitochondrial-inducing apoptosis factor, which is required for maintenance of functional mitochondrial respiratory chain protein complexes, resulted in improved insulin sensitivity and glucose uptake (Pospisilik et al., 2007). Altogether, this emphasizes a possible compensatory increase in glycolytic energy metabolism immediately following reduced energy production due to BPA-induced mitochondrial dysfunction.

BPA is an endocrine disruptor and has been shown to imitate E2 in vivo (Alonso-Magdalena et al., 2006). Acute exposure to BPA or E2 in mice has been shown to reduce glycemia and increase plasma insulin (Alonso-Magdalena et al., 2006). Therefore, we used the estrogen receptor antagonist ICI to determine whether the effects at 10^5 nM of BPA in L6 myotubes were through the estrogen receptor. We found that at 10^5 nM, BPA-induced increase in glucose uptake was reversed by ICI. Interestingly, however, ICI was unable to reverse the negative effects of BPA on mitochondrial function or glycolysis. BPA has been shown to affect metabolism through different classical and nonclassical estrogen receptors. For example, in the study by Alonso-Magdalena et al. (2006), they showed that ICI was able to reverse increased pancreatic insulin content, however, ICI had no effect of serum glucose levels or insulin release in mice treated acutely with BPA. Therefore, it is likely that the

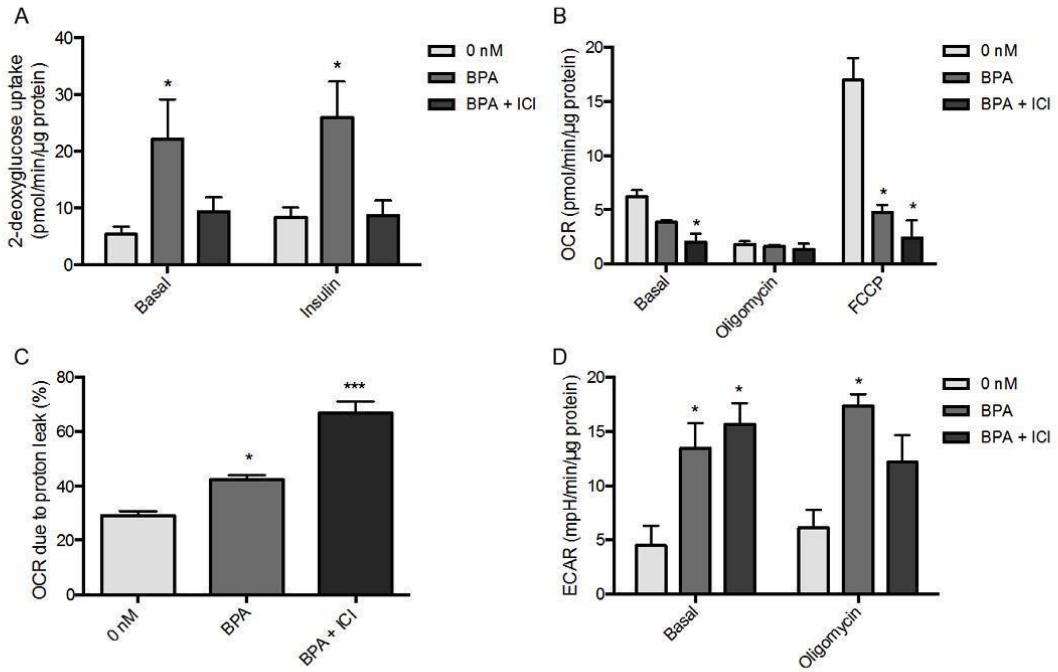

Fig. 4. The effects of ICI on BPA-induced glucose uptake and mitochondrial dysfunction in L6 myotubes. L6 myotubes were treated with vehicle (DMSO), BPA (10^5 nM), or BPA (10^5 nM) and ICI (10 nM) during the last 24 h of differentiation. (A) Uptake of 2-deoxyglucose in L6 myotubes in the basal condition (no insulin) or treated with insulin (100 nM) for 20 min. (B) Basal OCR was first measured followed by treatments with: oligomycin (3 µM) to assay for proton leak, and FCCP (2 µM) to assay maximal OCR. n = 4, each independent experiment was done in 3–5 replicates. (C) Percent of basal OCR due to proton leak (n = 4). (D) Basal and maximal (oligomycin-treated) ECAR. Data represent mean ± SEM. *p < 0.05 compared to vehicle. (A–C: one way-ANOVA, D: two-way-ANOVA).

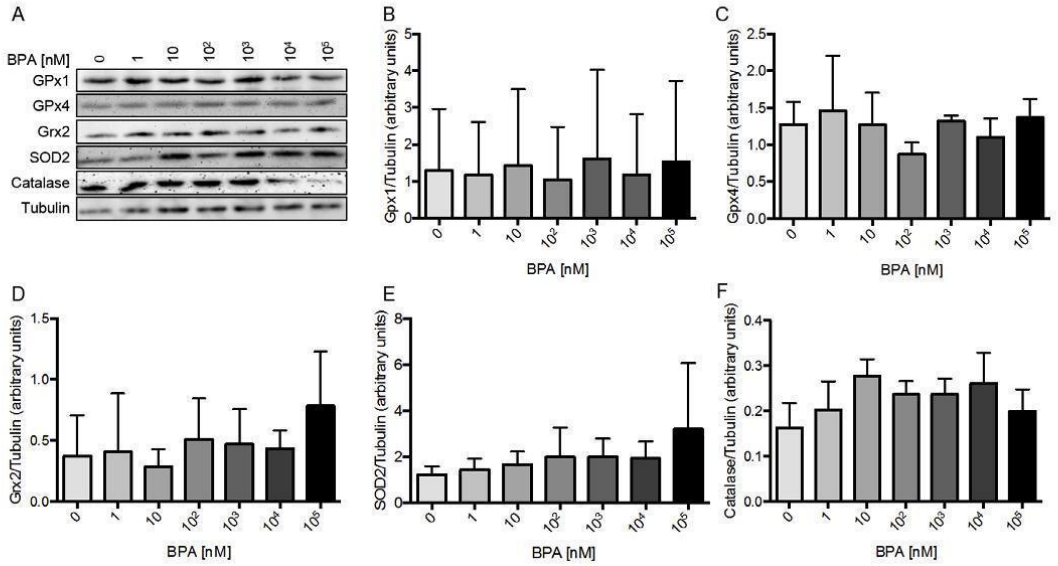

Fig. 5. The effects of 24-h BPA treatment on oxidative stress markers in L6 myotubes. (A) Representative Western blot of the oxidative stress markers GPx1 and 4, Grx2, SOD2, and catalase. Tubulin was used as a loading control. (B–F) Quantification of GPx1 (B), GPx4 (C), Grx2 (D), SOD2 (E), and catalase (F) by density analysis (n = 3 independent experiments). Data represent mean ± SEM (one-way ANOVA).

effects of BPA in our study were through classical and possibly non-classical estrogen receptors pathways.

Mitochondria are a major source of reactive oxygen species (ROS) production and oxidative stress (Murphy, 2009). Increased oxidative stress is often a result of mitochondrial dysfunction; including mitochondrial DNA damage, protein aggregation and lipid peroxidation, which can eventually result in mitophagy and/or apoptosis (Montgomery and Turner, 2015). In our study, we found no effect of acute BPA exposure on levels of several oxidative stress markers such as GPx4, GPx1, Grx2, catalase, and SOD2. It is likely that acute, 24-h BPA treatment is not long enough to induce alterations in oxidative stress marker levels. Thus, future studies should determine the effects of chronic BPA exposure on oxidative stress in skeletal muscle. Furthermore, muscle mitochondrial dysfunction can be induced by many other factors, and is not necessarily the result of ROS production (Mogensen et al., 2007). Nonetheless, proton leak measured after ATP synthase inhibition by oligomycin treatment was significantly increased under high BPA exposure. Mitochondrial proton leak, resulting from decreased mitochondrial membrane potential, is believed to decrease mitochondrial ROS production to protect cells from oxidative stress (Mailloux and Harper, 2011). This suggests the possibility that increased ROS production following BPA exposure was counteracted by increased proton leak in order to protect cells against BPA-induced oxidative stress.

Exposure to BPA can occur through a variety of routes, including oral consumption, respiration, and transdermal absorption (Genuis et al., 2012). Dietary consumption and transdermal exposure (through products such as thermal receipts) are the most common forms of exposure, with environmentally relevant doses in the low nanomolar range (Genuis et al., 2012). After exposure to BPA, there is low continuous release of BPA that can distribute to the body's storage; which is ultimately released into the urine (Calafat et al., 2008). This highlights that BPA exposure occurs most commonly in a chronic manner; therefore, future studies should investigate the effects of chronic BPA exposure in skeletal muscle (Calafat et al., 2008). In our study, we used a wide range of BPA concentrations since the effects of BPA have been shown to follow a non-monotonic dose response. Furthermore, it is important to note that despite low-dose exposure to BPA, large amounts can be released into the environment and contaminate groundwater and soil (Corrales et al., 2015). Although the levels we used for our study encompassed environmentally relevant concentrations and high concentrations that are rarely encountered environmentally, BPA levels above what is considered “environmentally relevant” may be achieved through biomagnification in wildlife, improper disposal, and sewage sludge (Corrales et al., 2015).

In this study, we treated L6 myotubes with BPA to determine the effects of this pollutant on muscle metabolism; there were however, some limitations. Although this provides insight into the effects of BPA on muscle metabolism, it excludes the effects of organ-organ crosstalk that occurs in vivo. Furthermore, short incubation with BPA represents the effects that occur after acute exposure, however it is likely that longer (chronic) incubations may better reflect what occurs in vivo. It is also important to note that although there is the possibility of cell culture contamination by BPA from cell culture material, it is unlikely that BPA is released from the cell culture materials as described by (Biswanger et al., 2006).

5. Conclusion

There is an increasing awareness on the effects of environmental pollutants on the development of metabolic diseases. Epidemiological studies have demonstrated that exposure to the xenobiotic, BPA, has been linked to the development of T2D (Shankar and Teppala, 2011). Therefore, we set out to determine the mechanism that characterizes the link between BPA exposure and the development of T2D. Taken all together, our study provides evidence that acute BPA exposure of skeletal muscle cells at environmentally relevant concentrations is not associated with altered mitochondrial function and glucose metabolism. However, at high concentrations of BPA (10^5 nM) there is mitochondrial dysfunction that may cause a compensatory shift from oxidative metabolism to glycolytic metabolism, through alterations in the insulin-signaling pathway. Furthermore, there is no parallel change in the levels of oxidative stress markers following acute BPA exposure. Therefore, it is unlikely that at environmentally-relevant doses, acute BPA exposure can be linked to the development of T2D through altered muscle metabolism. This work highlights the importance of establishing whether these effects could be modeled in vivo, since there is the possibility that BPA could also affect muscle mitochondrial function and glucose metabolism through inter-organ cross talk.

Author contributions

Experiments were performed in Dr. Aguer's laboratory at the Institut du Savoir Monfort (Ottawa, ON, Canada) except for Seahorse and radiation experiments that were conducted in Dr. Harper's laboratory (Biochemistry, Microbiology, and Immunology Department, University of Ottawa, Canada). Conception and design of the experiments were done by CA; collection, assembly, analysis, and interpretation of data by FA, LC, LG, AC, and CA; drafting the article or revising it critically for important intellectual content by FA, LC, LG, AC, and CA; and approval of the final version by FA, LC, LG, AC and CA.

Conflicts of interest

The authors have no competing interests to report and have no potential or real conflicts of interest to declare.

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CRediT authorship contribution statement

Fozia Ahmed: Validation, Methodology, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Visualization. **Lucia Chehadé:** Validation, Methodology, Formal analysis, Investigation, Writing - original draft, Writing - review & editing. **Léa Garneau:** Validation, Methodology, Investigation, Writing - review & editing. **Audrey Caron:** Validation, Methodology, Investigation, Writing - review & editing. **Céline Aguer:** Conceptualization, Methodology, Resources, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mce.2019.110580.

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- Researching the effects of environmental pollutants (bisphenols) on the development of metabolic disease

08/2019 - 12/2019 — **Visiting Researcher, Clinical Diabetes and Metabolism**
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ACADEMIC ACTIVITY

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03/2020 Caron, A., **Ahmed, F.,** Peshdary, V., Garneau, L., Atlas, E., and Aguer, C..
Adipose-to-muscle communication explains abnormal muscle glucose uptake with
PCB126 exposure. Submitted: March 2020, Environmental Health Perspectives

Manuscript evaluation

01/2020 Environmental Toxicology and Pharmacology. Drafted the first evaluation of the manuscript and comments were finalized by supervisor.

Conferences

05/2020 **International Conference on Environmental Research and Public Health**, Naples, Italy (postponed)
Poster Title: Plastics and Human Health: The Effects of Bisphenol A on Adipose Tissue Metabolism

04/2019 **6th Canadian Obesity Summit,** Ottawa, Canada
Poster Title: The direct effects of 24-hour treatment of BPA on muscle mitochondrial function and insulin sensitivity in L6 cells

Oral Presentations

12/2019 **IMV Seminar,** Uppsala University, Sweden
Invited speaker at Department of Medical Sciences (IMV)
Plastics and human health: exposure to chemicals in plastics and the development of metabolic disease

09/2019	**Seminar in the Clinical Diabetes and Metabolism** (KDM) group, Uppsala University, Sweden The effects of BPA and BPS on muscle metabolism and cross-talk with adipose tissue
04/2019	**Institut du Savoir Montfort (ISM) Conference Day** Oral presentation: Effects of pollutants on skeletal muscle insulin sensitivity and crosstalk with adipose tissue

Teaching

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2014-2018	**Deans Honors List** (University of Western Ontario) ● Awarded to full-time students who earn a minimum average of 80%
2014-2018	**Western Scholars** ● Honors society for students entering university with a minimum of 90% and maintaining an average of at least 80%
2014	**The Governor General Academic Medal Recipient**, 2014 ● Awarded to the student with the highest academic average upon graduation

SCHOLARSHIPS

2019 **Mitacs Globalink Research Award**
- Grant awarded to students to conduct research abroad

2018 **University of Ottawa Entrance Scholarship**
- Awarded to students based on academic excellence with a minimum entrance average of 80%

2017 **OBW Summer Research Scholarship,** 2017
- Scholarship awarded on a competitive basis to students based on academic's, extracurricular and a letter of nomination from university professor

2014 **Western University Continuing Scholarship**, 2014-2018
- Scholarship awarded to entering students who have achieved a minimum average of 95%

CERTIFICATES AND VOLUNTEERING

2019 - 2020 **uOttawa Global Recognition Certificate**
2019 - 2019 **Biochemistry Tutor**
2015 - 2018 **Academic Tutor**: Learning Disability Association of London Region
2016 - 2017 **Mentor**: Big Brothers Big Sisters: Go Girls