Bisphenol A effects on gene expression in adipocytes from children: association with metabolic disorders

Ciro Menale^{1,2,†}, Maria Teresa Piccolo^{2,3,†}, Grazia Cirillo⁴, Raffaele A Calogero⁵, Alfonso Papparella⁴, Luigi Mita^{2,6}, Emanuele Miraglia Del Giudice⁴, Nadia Diano^{1,2}, Stefania Crispi^{2,3,*} and Damiano Gustavo Mita^{2,6,*}

¹Department of Experimental Medicine, Second University of Naples, Via S. Maria di Costantinopoli 16, 80138 Naples, Italy

²National Laboratory of Endocrine Disruptors, INBB, Via P. Castellino 111, 80131 Naples, Italy

³Gene Expression and Molecular Genetics Laboratory, IBBR – CNR, UOS Napoli Via P. Castellino 111, 80131 Naples, Italy

⁴Department of Woman, Child and General and Specialized Surgery, Second University of Naples, Via Luigi De Crecchio 4, 80138 Naples, Italy

⁵Bioinformatics and Genomics Unit, MBC Molecular Biotechnology Center, University of Turin, Via Nizza 52, 10126 Turin, Italy

⁶Biophysics Laboratory, IGB – CNR, Via P. Castellino 111, 80131 Naples, Italy

*S Crispi and D G Mita contributed equally as co-last authors

[†]C Menale is now at IRGB – CNR UOS Milano c/o Humanitas Clinical Institute Research Center, Via Manzoni 113, 20089 Rozzano, Milano, Italy

[†]M T Piccolo is now at Department of Environmental, Biological and Pharmaceutical Sciences and Technologies, Second University of Naples, Via Vivaldi 43, 81100 Caserta, Italy

Correspondence
should be addressed
to S Crispi
Email
stefania.crispi@ibbr.cnr.it

Abstract

Bisphenol A (BPA) is a xenobiotic endocrine-disrupting chemical. *In vitro* and *in vivo* studies have indicated that BPA alters endocrine-metabolic pathways in adipose tissue, which increases the risk of metabolic disorders and obesity. BPA can affect adipose tissue and increase fat cell numbers or sizes by regulating the expression of the genes that are directly involved in metabolic homeostasis and obesity. Several studies performed in animal models have accounted for an obesogen role of BPA, but its effects on human adipocytes – especially in children – have been poorly investigated. The aim of this study is to understand the molecular mechanisms by which environmentally relevant doses of BPA can interfere with the canonical endocrine function that regulates metabolism in mature human adipocytes from prepubertal, non-obese children. BPA can act as an estrogen agonist or antagonist depending on the physiological context. To identify the molecular signatures associated with metabolism, transcriptional modifications of mature adipocytes from prepubertal children exposed to estrogen were evaluated by means of microarray analysis. The analysis of deregulated genes associated with metabolic disorders allowed us to identify a small group of genes that are expressed in an opposite manner from that of adipocytes treated with BPA. In particular, we found that BPA increases the expression of pro-inflammatory cytokines and the expression of *FABP4* and *CD36*, two genes involved in lipid metabolism. In addition, BPA decreases the expression of *PCSK1*, a gene involved in insulin production. These results indicate that exposure to BPA may be an important

Key Words

- bisphenol A
- adipocytes
- children
- gene expression
- metabolic homeostasis

risk factor for developing metabolic disorders that are involved in childhood metabolism dysregulation.

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Introduction

Bisphenol A (BPA) is the key monomer in the production of the polycarbonate and epoxy resins that are used for many food containers, such as cans or bottles for children (Halden 2010). BPA belongs to the class of endocrine disruptor chemicals (EDCs) that alter the functions of the endocrine system by competing with endogenous hormones. For humans, the main exposure to BPA occurs through the diet, with the intake of contaminated food and drinking water (Kang *et al.* 2006). BPA intake is higher and more dangerous in infants and children, and it can lead to the onset of several diseases, such as cancer, endometriosis, birth defects, and developmental and neuronal disorders (Rochester 2013, Delclos *et al.* 2014). BPA intake has also been associated with obesity and metabolic disorders (Rochester 2013, Lakind *et al.* 2014), specifically in children (Trasande *et al.* 2012, Lee *et al.* 2013, Nicolucci *et al.* 2013).

BPA is known to have estrogen-like properties, but some evidence has also shown that at low environmentally relevant doses, it can act as an estrogen antagonist by exerting an opposite effect, which suggests that BPA could act also through non-classical ER pathways (Li *et al.* 2012, Boucher *et al.* 2014).

In vivo and *in vitro* studies have confirmed that BPA exerts its disrupting effects on the classical nuclear receptors estrogen receptors alpha and beta (ER α and ER β), non-classical membrane estrogen receptor (ncmER), estrogen-related receptor gamma (ERR γ), G protein-coupled receptor 30 (GPR30), and the aryl hydrocarbon receptor (AhR) (Hugo *et al.* 2008, Alonso-Magdalena *et al.* 2012). Through these interactions, BPA is thought to be involved in the onset of metabolic dysfunction. Given the lack of data in human subjects, more studies on molecular effects are needed in order to shed light on any association BPA may have with metabolic diseases, particularly in childhood obesity.

Currently, a large amount of attention has been paid to analyzing the BPA disrupting action in modulating endocrine functions of human adipose tissue. A recent study (Valentino *et al.* 2013) demonstrated a reduced insulin-stimulated tyrosine phosphorylation of insulin receptors in adult adipocytes treated with BPA and a

consistent reduction of insulin downstream signaling. Another study (Wang *et al.* 2013) reported that environmentally relevant BPA concentrations in adipocytes from children increase the expression and the enzymatic activity of 11 β -hydroxysteroid dehydrogenase type 1. This is a key enzyme in adipocyte differentiation and lipid synthesis. These results support the hypothesis that BPA could stimulate preadipocyte differentiation and adipogenesis and thus promote obesity in childhood.

Moreover, molecular studies have demonstrated that a large number of genes that are modulated by BPA induce negative health effects (Singh & Li 2012). Specifically, BPA exposure during adipocyte differentiation affects gene expression and subsequently modulates adipose tissue functions. Examples include PPAR, C/EBP, LPL, GLUT4, CYP19, GPAT and DGAT, and Leptin (OB) (Vom Saal *et al.* 2012, Boucher *et al.* 2014, Ohlstein *et al.* 2014). BPA also increases lipoprotein lipase gene expression and the corresponding enzymatic activity that leads to triacylglycerol accumulation (Masuno *et al.* 2002). BPA has also been found to increase glucose transporter GLUT4 levels and thus to alter glucose uptake (Masuno *et al.* 2002, Sakurai *et al.* 2004). All of these aspects indicate that BPA exposure, by affecting gene expression, directly regulates adipocyte function and metabolism homeostasis.

In this paper, we describe the role of BPA in modulating canonical endocrine function, which regulates metabolism during childhood. We used differentiated mature adipocytes obtained from non-obese children to evaluate if environmentally relevant doses of BPA interfere with estrogen function in metabolism.

Genes associated with metabolism and those modulated by estrogen in adipocytes were selected by means of transcriptional analysis. Subsequently, the gene expression and physiological outcomes were further analyzed following BPA treatment.

The results indicated that environmentally relevant BPA doses induce changes in gene expression that are likely to affect their activity. Deregulated genes are potentially involved in metabolic disorders through the possible impairment of pancreatic functions.

Materials and methods

Chemicals and reagents

Culture media, fetal bovine serum (FBS), penicillin, streptomycin, and L-glutamine were obtained from Euroclone (Milan, Italy). BPA, 17 β estradiol (E₂), collagenase type 1, human insulin, recombinant human epidermal growth factor (rEGF), and oil red O (ORO) powder were purchased from Sigma–Aldrich. Trizol reagent and a Human Interleukin 1 Beta (IL1B) ELISA kit were obtained from Invitrogen Life Technologies. High-Capacity cDNA Reverse Transcription kit was purchased from Applied Biosystems. A GeneChip Human Gene 1.0 ST array was obtained from Affymetrix (Santa Clara, CA, USA). IL18 and CCL20 ELISA kits and a Triglyceride Quantification kit were obtained from Abcam (Cambridge, UK). A Human Insulin ELISA kit was purchased from Millipore (St. Charles, MO, USA).

Patients and cell culture

This study was approved by the ethical committee of the Second University of Naples. Informed consent was obtained by the parents and, where appropriate, by the children involved. All of the patients underwent orchidopexy surgery. For all of the samples, subcutaneous adipose tissue was collected from the inguinal area, which is a natural deposit of adipose tissue. A total of eight preadipocyte samples were *in vitro* differentiated in mature adipocytes: five samples were used for microarray analysis, and all of them were used for the subsequent biological validations (Table 1).

Human preadipocytes and differentiated mature adipocytes were cultured in phenol red free DMEM supplemented with 10% (v/v) FBS, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 1 mM L-glutamine. Preadipocytes were grown with 10 ng/ml rEGF. Subcutaneous adipose tissue fragments were obtained from eight non-obese children within 30 min after starting elective

surgery. Adipose tissues were washed in culture medium for 1 h at 37 °C and then digested using 1 mg/ml collagenase type 1 for 60 min at 37 °C. Dissociated tissue was pelleted at 1200 g for 10 min, and the stromal-vascular cells and preadipocytes were resuspended in 160 mM NH₄Cl to remove erythrocytes. Cells were filtered (through 100 μ m nylon mesh), and the purified preadipocytes were seeded in 60 mm cell culture dishes. These cells were differentiated at passage IV. Differentiation into adipocytes was induced as previously described (Masuno *et al.* 2005). Adipocytes were cultured in medium supplemented with 10 μ g/ml insulin. BPA treatments were done 24 h after the differentiation and culturing of adipocytes without insulin. Insulin was removed from the medium to avoid any effects of insulin stimuli on the gene expression.

Human pancreatic cell line PANC-1 was purchased from ATCC (<http://www.atcc.org>) and cultured in high-glucose DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μ g/ml streptomycin. PANC-1 cells were cultured in high-glucose medium (4.5 g/l) to stimulate insulin secretion. Cells were expanded up to passage X before use.

Cells were grown in a humidified atmosphere containing 5% CO₂ at 37 °C. Cells that had been treated with vehicle only – 0.1% (v/v) ethanol – were used as the control.

Experiments were performed in triplicate for each sample.

Cell treatments

Mature adipocytes and PANC-1 human pancreatic cells were treated for 24 h with different BPA concentrations (1, 10, and 100 nM) and/or E₂ (1 nM) that were formed by diluting 100% ethanol stock solutions starting from 1, 10, and 100 μ M, BPA and 1 μ M E₂ directly in the culture medium.

Table 1 Clinical sample characteristics

Case no.	Age	Surgery	BMI	Array	qPCR	Biological validation
1	7	Orchidopexy	19		X	X
2	10	Orchidopexy	20	X	X	X
3	8	Orchidopexy	19	X	X	X
4	8	Orchidopexy	20	X	X	X
5	9	Orchidopexy	21		X	X
6	9	Orchidopexy	20	X	X	X
7	8	Orchidopexy	20	X	X	X
8	10	Orchidopexy	19		X	X

GeneChip array sample preparation and data analysis

Microarray analysis was performed on *in vitro*-differentiated adipocytes of five non-obese children. In order to identify the estrogen transcriptional modifications, cells were treated for 24 h with 1 nM E₂. Cells that had been treated with vehicle only were used as the control. Total RNA was extracted using Trizol reagent. Biotinylated cRNA target preparation and target hybridization were performed according to Affymetrix instructions. Experiments were performed using GeneChip Human Gene 1.0 ST arrays. All of the hybridizations, washing, staining, and scanning procedures were done using a GeneChip station (FS 450, Scanner 3000, Affymetrix), according to the manufacturer's instructions. Laser scanning generated CEL files.

Two prototypic situations – untreated and E₂-treated cells – were analyzed to generate background-normalized image data. Data were analyzed using a ChannelGUI Bioconductor package. Gene-level calculations were performed by Robust Multichip Average, and normalization was achieved by quantile sketch, as previously described (Crispi 2013). To assess differential expression at the gene-level, an empirical Bayes method (Smyth 2004) together with a false discovery rate (FDR) correction of the *P* value (Westfall & Young 1993) were used. Differentially expressed genes were generated using an FDR of ≤ 0.05 and an absolute log₂-(fold-change) threshold of 1. Microarray data were deposited in the Gene Expression Omnibus database (GEO accession no. GSE58516).

Deregulated genes were functionally classified through an IPA Core Analysis (IPA 7.0, Ingenuity Systems, Redwood City, CA, USA). IPA associates molecules under analysis with known biological functions and diseases. The link between a category and its associated molecules is defined by a *P* value. In general, the more molecules that are involved, the more likely it is that the association is not a result of random chance, and thus the more significant the *P* value.

Quantitative RT-PCR analysis

Total RNA (200 ng) from each adipocyte sample that had been untreated or treated with BPA or E₂ was converted to cDNA using a High-Capacity cDNA Reverse Transcription kit under the conditions described by the supplier. Gene specific primers for the selected genes (Table 2) were designed at exon–exon junction using Primer Express 2.0 software (Applied Biosystems). Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as an internal control. The entire procedure for

Table 2 Genes amplified by RT-PCR and sequences of the primers used for their amplification

Gene		Primer sequence
<i>ESR1</i>	Forward	GATGAAAGGTGGGATACGAAAAGA
	Reverse	CTCTGGCGCTTGTGTTTCAA
<i>ESR2</i>	Forward	AAGAAGATTCCCGGCTTTGTG
	Reverse	AGCTCAGCCTGTTCG
<i>ESRRG</i>	Forward	GCCAGCCAAAAAGCCATATAAC
	Reverse	GGCATAGATCTTCTCCGGTTCA
<i>GPER</i>	Forward	ACGAGACTGTGAAATCCGCAACCA
	Reverse	ATCAGGCTGGAGGTGCACCTGGAA
<i>Leptin (OB)</i>	Forward	TCACCAGGATCAATGACATTTC
	Reverse	CCAAACCGGTGACTTTCTGTTT
<i>FABP4</i>	Forward	TTGACGAAGTCACTGCAGATGA
	Reverse	CAGGACACCCCATCTAAGGT
<i>IL1B</i>	Forward	CTGAGCTGCCAGTGAAATG
	Reverse	TCAGCTTCAAAGAACAAGTCATCCT
<i>IL8</i>	Forward	GAGTGATTGAGAGTGGACCACT
	Reverse	AGAGCTCTCTCCATCAGAAAGCT
<i>IL18</i>	Forward	GCTGAACCAGTAGAAGACAATTGC
	Reverse	AGCTTGCCAAAGTAATCTGATTCC
<i>CCL20</i>	Forward	CCACCTCTGCGGCGAAT
	Reverse	CTGTGTATCCAAGACAGCAGTCAA
<i>CCL13</i>	Forward	GCAGAGGCTGAAGAGCTATGTGA
	Reverse	CTCCTTGCCAGTTTGGTTCT
<i>CD36</i>	Forward	TGAAGAAGAACATAGGACATACTTGA
	Reverse	TGCAGCCGTTTTGCAAATT
<i>PCSK1</i>	Forward	TCTTCAATGATCCCATGTGGAA
	Reverse	GCAGGGCTGCCGTCATC

quantitative RT-PCR (qRT-PCR) analysis – including primer design, reactions, amplicon specificity, and determination of gene target expression levels – was performed as previously described (Crispi *et al.* 2009).

Lipid area and triglyceride content

Cytoplasmic lipid accumulation was determined by ORO staining, as previously described (Kuri-Harcuch & Green 1978). Cells fixed with 10% (v/v) formalin in PBS were stained for 40 min at 37 °C. Analysis of the mean lipid area was performed using Image J software (Rasband, W.S., ImageJ, National Institutes of Health, Bethesda, MD, USA, <http://imagej.nih.gov/ij/>, 1997–2012), and five pictures were analyzed for each experiment.

Triglyceride measurement was done following the kit manufacturer's protocol.

Measurement of cytokines and insulin

IL1B, IL18, and CCL20 in adipocytes and insulin levels in PANC-1 cell culture medium were determined by ELISA following the manufacturer's instructions. Data were normalized to the total cell number used for the experiments.

Statistical analysis

Statistical analyses were performed using Prism 4.0 software (GraphPad Software, San Diego, CA, USA). Treatment efficacy and differences between multiple treatments were determined by ANOVA test and *post hoc* Bonferroni's correction. An unpaired *t*-test was used for analyzing a comparison of the two groups. A value of $P < 0.05$ was considered statistically significant. All data are shown as mean $\pm$ S.E.M. or mean $\pm$ S.D.

Results

Gene profiling and pathways analysis

To analyze the role of BPA in modulating the canonical endocrine function involved in metabolic homeostasis at the molecular level, we first evaluated the pattern modifications that were primed by estrogen and associated with metabolism. To this end, we performed transcriptional profiling by exposing human *in vitro*-differentiated mature adipocytes obtained from male non-obese, prepubertal children to E_2 . Adipocytes were treated with 1 nM E_2 , a concentration that was previously reported as the minimum dose needed to achieve BPA-similar effects on adiponectin suppression in adipose tissue from human breast explants (Hugo *et al.* 2008). This concentration is also known to activate *in vitro* ERs (Li *et al.* 2012).

Preadipocytes from five samples were *in vitro* differentiated in mature adipocytes as described in the Materials and methods section, and ORO staining was used as a control of the correct differentiation. Cell differentiation was induced for 72 h to allow us to observe the morphological change in the fibroblast-like phenotype. 24 hours after differentiation, the cells exhibited the typical morphological characteristics of mature adipocytes, having accumulated red lipid droplets in the cytoplasm (Supplementary Figure 1, see section on supplementary data given at the end of this article).

Microarray analysis was performed using a Human Gene 1.0 ST arrays that interrogates 28 869 well-annotated genes. Differentiated adipocytes were treated for 24 h with E_2 before processing. Vehicle-only treated cells were used as a control.

Gene-level expression profiling was detected by linear model statistics using an empirical Bayes method to moderate the standard errors. To evaluate transcriptional modifications, E_2 -treated cells were compared to control cells. To identify differentially expressed genes, an

absolute $\log_2$ -fold change $\geq \pm 1$ and a P value of ≤ 0.05 were applied as cutoffs. Following this method, a total of 29 differentially expressed genes were selected from the E_2 -treated cells.

This small number is probably a result of the high background noise related to the number and kind of samples. In fact, it should be remembered that a relatively small sample size shows a high degree of variability. This variability can be the result of combined influences, both genetic and nutritional.

A complete list of differentially expressed genes in the E_2 -treated cells is reported in Supplementary Table 1, see section on supplementary data given at the end of this article. These genes were functionally classified through an IPA Core Analysis (IPA 7.0, Ingenuity System) that associates molecules with known biological functions and diseases. The link between a category and the associated molecules is defined by a P value. Significant P values indicate that the molecules–function association is not the result of random chance.

The analysis showed that most of these genes were associated with diseases and biofunctions that were mainly related to inflammation, carbohydrate and lipid metabolism, cell signaling, and metabolic diseases with P values that ranged from 2.84×10^{-6} to 1.66×10^{-3} (Fig. 1A). This was expected, as it is well known that estrogens are associated with metabolism regulation and inflammation.

To find functional relationships among the genes that are specifically involved in metabolic disorders, we performed an IPA pathway analysis. The resulting pathway highlighted several closely linked genes that are related to obesity and metabolic diseases. In particular, we found some pro-inflammatory cytokines, such as chemokine (C-C motif) ligand 13 (*CCL13*), chemokine (C-C motif) ligand 20 (*CCL20*), interleukin 18 (*IL18*), and IL1 beta (*IL1B*), associated in the pathway. Overproduction of these molecules within the adipose tissue has been associated with the development of insulin resistance (Odegaard *et al.* 2007). We also found associated in the pathway CD36 molecule (*CD36*) and fatty acid binding protein 4 (*FABP4*), two molecules that are involved in lipid binding and transport, and proprotein convertase subtilisin/kexin type 1 (*PCSK1*), which is involved in the active production of insulin (Fig. 1B and C). The results of the associated genes in this pathway were very interesting, because they could play a key role in metabolic disorders depending on how they are expressed.

Figure 1

IPA functional analysis. (A) Functional classification showed that most of the genes are associated with diseases and biofunctions that are mainly related to inflammation, carbohydrate and lipid metabolism, cell signaling, and metabolic diseases. A class is considered significant when the P value is above the threshold. (B and C) Functional relationship between

deregulated genes related to metabolic disorders and their relative qRT-PCR expression after E₂ (B) or BPA (C) treatment. Red indicates up-regulated genes, whereas green indicates down-regulated genes. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JME-14-0282>.

Validation of gene expression and BPA effects on selected genes

To independently validate the expression of the genes highlighted in the pathway, we used qRT-PCR. Furthermore, to investigate whether BPA could have an estrogen-like or an estrogen-antagonist action in adipocytes, we checked their expression after E₂ or BPA treatment.

To this end, a preliminary experiment was performed to identify the most effective BPA concentration for the modulation of gene expression. Preadipocytes from eight non-obese, prepubertal children were *in vitro* differentiated into mature adipocytes as described

above and were treated in a time-course analysis (12, 24, and 48 h) with different environmental doses of BPA exposure (1, 10, and 100 nM) (Hugo *et al.* 2008). BPA concentrations were chosen by taking into account previous studies that described the concentrations that were able to affect metabolic homeostasis (Hugo *et al.* 2008), the BPA ranges found in adult and fetal serum (Welshons *et al.* 2006), and the range of BPA concentrations detected by our group in children's urine (Nicolucci *et al.* 2013).

Gene modulation exerted by BPA in adipose tissue was assessed by measuring the expression of

Figure 2

BPA gene modulation in adipose tissue. The effect of different BPA concentrations (1, 10, or 100 nM) at different times (12, 24, or 48 h) on the expression of (A) ER α , (B) ER β , (C) ERR γ , (D) GPR30, and (E) leptin in mature adipocytes derived from preadipocytes from children. E₂ treatment (1 nM)

was used to compare BPA effects to the effects of canonical hormones. Data represent mean fold change $\pm$ s.d. relative to the untreated cells *P < 0.05, **P < 0.01, ***P < 0.001 as compared to the control.

estrogen-responsive receptors that are known to interact with BPA and are expressed in human adipose tissue, including: estrogen receptor alpha (*ESR1*, ER α), estrogen receptor beta (*ESR2*, ER β), estrogen-related receptor gamma (*ESRRG*, ERR γ) and G protein-coupled receptor 30 (*GPER*, GPR30). Their expression supports the suggestion that adipose tissue is potentially BPA responsive (Hugo *et al.* 2008, Vom Saal *et al.* 2012).

Figure 2A shows that ER α is up-regulated by low doses of BPA stimulation. Specifically, 10 and 100 nM BPA are the most effective doses for modulating ER α gene expression. No difference was observed after E₂ treatment.

No changes were found in ER β mRNA levels after BPA exposure (Fig. 2B). Figure 2C shows that ERR γ mRNA levels are up-regulated after 10 nM BPA or E₂ stimulation. Both ER α and ERR γ expression data show a typical trend of inverted U dose response being tightly linked to BPA exposure that has also been described by other studies (Boucher *et al.* 2014, Liang *et al.* 2014, Vandenberg 2014). The expression of GPR30 specifically decreased after the 10 nM BPA treatment (Fig. 2D) and supports the dual activity of BPA in the signaling pathway, because it can act through either a classical or a non-classical estrogenic pathway (Sharma *et al.* 2013).

The modulation of ER α expression indicates that BPA might directly alter the estrogen signaling pathways with antagonist activity, whereas ERR γ expression appeared similar in both the BPA and E₂ treatments.

The obesogenic activity of BPA was further demonstrated by the expression of leptin, one of the most important adipose-derived hormones, which plays a key role in regulating energy homeostasis, appetite, and metabolism and acts directly in the hypothalamus (Brennan & Mantzoros 2006). Figure 2E indicates that Leptin mRNA levels in mature adipocytes from children were up-regulated following the 10 and 100 nM BPA treatments as compared to the control cells.

These results allowed us to choose 10 nM as the BPA concentration and 24 h as the time treatment for the subsequent experiments. Thus, qRT-PCR was used to compare gene expression levels in adipocytes after E₂ or BPA treatment. As shown in Table 3, qRT-PCR mostly confirmed the expression trend that was detected by microarray analysis after E₂ treatment. Surprisingly, BPA treatment resulted in an opposite expression for most of the genes (Table 3 and Fig. 3B), which suggests that the accumulation of BPA in the adipose tissue could affect the normal endocrine functions of mature adipocytes.

BPA promotes inflammation and lipid accumulation

To test whether the gene expression changes caused by BPA in adipocytes could have a physiological and/or functional significance, further experiments were performed to see if the biochemical responses were consistent with our hypothesis.

qRT-PCRs showed that BPA induces the expression of the pro-inflammatory genes *CCL20*, *IL18*, and *IL1B*.

Table 3 Differentially deregulated genes associated with metabolic disorders in E₂-treated adipocytes and qPCR expression after E₂ or BPA treatment. qPCR values are expressed as mean $\pm$ s.d. as reported in the columns

Gene	Description	E ₂		BPA	Association with metabolic disorders
		Array fold change ^a	qPCR ^b	qPCR ^b	
<i>CCL13</i>	Chemokine (C-C motif) ligand 13	1.48	1.14 $\pm$ 0.5	1.957 $\pm$ 0.8	Hashimoto <i>et al.</i> (2006)
<i>CCL20</i>	Chemokine (C-C motif) ligand 20	–2	2.118 $\pm$ 0.7	2.086 $\pm$ 0.7	Duffaut <i>et al.</i> (2009) and Villaret <i>et al.</i> (2010)
<i>CD36</i>	CD36 molecule	–2.488	–1.35 $\pm$ 0.4	2.047 $\pm$ 0.6	Hajri <i>et al.</i> (2002), Pravenec <i>et al.</i> (2003) and Rac <i>et al.</i> (2007)
<i>FABP4</i>	Fatty acid binding protein 4	–2.401	–1.04 $\pm$ 0.2	1.306 $\pm$ 0.3	Hardaway & Podgorski (2013)
<i>IL18</i>	Interleukin 18	1.586	–1.31 $\pm$ 0.2	1.575 $\pm$ 0.5	Banerjee & Saxena (2012)
<i>IL1B</i>	Interleukin 1 beta	–1.376	–1.7 $\pm$ 0.2	2.17 $\pm$ 0.7	Ouchi <i>et al.</i> (2011) and Esser <i>et al.</i> (2013)
<i>IL8</i>	Interleukin 8	–2	1.28 $\pm$ 0.8	–1.562 $\pm$ 0.5	Samaras <i>et al.</i> (2010) and Banerjee & Saxena (2012)
<i>PCSK1</i>	Proprotein convertase subtilisin/kexin type 1	1.848	1.80 $\pm$ 0.2	–1.366 $\pm$ 0.2	Creemers <i>et al.</i> (2012) and Martin <i>et al.</i> (2013)

^aPerformed on five samples.

^bPerformed on eight samples.

Therefore, we checked the secretion of deregulated cytokines IL1B, IL18, and CCL20 in adipocyte culture medium after a 24 h treatment with 10 nM BPA. As shown in Fig. 3, all of these inflammatory cytokines were released at higher levels in the medium with BPA-treated cells. In particular, the amount of secreted IL1B was about three times higher than it was in control cells: 14.4 pg/10⁶ cells vs 4.17 pg/10⁶ cells; the amount of secreted IL18 was about two times higher than it was in control cells: 20.16 pg/10⁶ cells vs 10.98 pg/10⁶ cells; and the amount of secreted CCL20 was about six times higher than it was in control cells: 18.74 pg/10⁶ cells vs 3.25 pg/10⁶ cells. These results confirm that BPA promotes inflammation in mature adipocytes.

We then assessed if the BPA-mediated dysregulation of *CD36* and *FABP4* – two genes involved in lipid binding and transport – could affect lipid metabolism by evaluating the cytoplasmic lipid accumulation. To this end, mature adipocytes were treated with 1, 10, and 100 nM BPA, and accumulation of lipid droplets were analyzed by determining the mean lipid area size of fat depots in mature adipocytes (Fig. 4A and B). The results showed a close correlation between BPA exposure and adipocyte hypertrophy, which is also correlated to BPA concentration (Fig. 4), with a mean lipid area that was significantly higher in the 1 and 10 nM BPA-exposed cells (36.35 and 52.47 μ m² respectively) as compared to the control cells (28.65 μ m²). Similar results were obtained when measuring the cellular triglyceride content, which was significantly increased after the BPA treatment (Fig. 4C).

BPA regulates insulin production

We also tested whether the *PCSK1* down-regulation that was observed after BPA treatment could result in a physiological outcome.

PCSK1 encodes for pro-protein convertase (PC1/3), an enzyme that is involved in pro-insulin cleavage and in subsequent insulin activation (Turpeinen *et al.* 2013).

Previous studies in rodents indicated that BPA directly affects pancreatic β -cell functions (Alonso-Magdalena *et al.* 2010, Wei *et al.* 2011), and a recent paper has demonstrated in a rat insulinoma cell line that BPA affects pancreatic function on insulin production by decreasing insulin production and secretion (Lin *et al.* 2013).

To evaluate whether BPA is linked to insulin production via *PCSK1* gene down-regulation, BPA effects were investigated in PANC-1 cells, which are widely used as a human model of pancreatic cells because of their ability to secrete insulin in response to high amounts of glucose in the culture medium (Hamil *et al.* 2008).

As shown in Fig. 5A, PANC-1 cells treated with 10 nM BPA secreted a total amount of active insulin that was significantly lower ($\sim$ 0.30 μ g insulin/10⁶ cells) than untreated cells ($\sim$ 0.45 μ g insulin/10⁶ cells).

Furthermore, this reduction is related to a decrease in the corresponding *PCSK1* mRNA after BPA exposure (Fig. 5B), which is in agreement with the results reported in Table 3.

It might be possible that BPA, by down-regulating the expression of *PCSK1*, could determine a reduction of the corresponding protein level (PC1/3) that in turn could be

Figure 3

Cytokine secretion. IL1B (A), IL18 (B), and CCL20 (C) secretion in culture medium after BPA treatment (10 nM) for 24 h, as determined by ELISA. Data are expressed as means $\pm$ S.E.M. of three independent experiments. * $P < 0.05$, ** $P < 0.01$ as compared to the control.

insufficient for cleaving pro-insulin and for generating active insulin.

Discussion

It is well known that BPA, acting as an endocrine disruptor, influences the endocrine system by competing with endogenous hormones, and its action is more harmful during development.

This study describes the molecular effects induced by *in vitro* BPA exposure on human primary adipocytes obtained from children.

The molecular effects by which BPA exerts its disrupting effects in adipose tissue during childhood are currently stimulating great interest in the scientific community (Bhandari *et al.* 2013, Eng *et al.* 2013, Harley *et al.* 2013). Most of the published studies analyze the effects of BPA on adipogenesis in murine 3T3-L1 cells or in human cell cultures obtained from adult adipose tissue (Masuno *et al.* 2005, Hugo *et al.* 2008, Valentino *et al.* 2013). A recent paper described the effects of BPA on adipogenesis using adipose cells from children as a model system (Wang *et al.* 2013).

In this study, we investigated how environmentally relevant doses of BPA affect the endocrine function of mature adipocytes differentiated *in vitro* and obtained from subcutaneous adipose tissue of non-obese, prepubescent male children. Sex was selected on the basis of our previous study, which showed that obese male children have higher BPA urinary levels than females (Nicolucci *et al.* 2013). Prepubescent children were selected because they are not influenced by relevant sex hormonal regulation. Non-obese children were chosen in order to eliminate any experimental bias resulting from the dysregulation of endocrine functions often found in overweight subjects.

To this end, we first analyzed transcriptional modifications in adipocytes exposed to estrogen, focusing our attention on molecules associated with metabolism. Statistical data analysis identified a small number of differentially expressed genes, due to the high sample noise related to the small size and high variability of the human samples. Deregulated genes were functionally characterized by IPA analysis, which specifically identified carbohydrate, lipid, and endocrine metabolic pathways as the main functional categories to which these genes belong. A further pathway analysis of the genes associated with metabolic disorders highlighted a functional pathway in which a small group of genes are interconnected. These genes are involved in adipose tissue inflammation, obesity, and metabolic diseases.

It is known that estrogens regulate cellular metabolism by programming gene expression in adipocytes and that they are implicated in metabolic disorder restoration. A lack of estrogens increases fat mass and impairs glucose tolerance and insulin resistance (Vom Saal *et al.* 2012).

To investigate whether BPA could mimic estrogen activity or antagonize E_2 function, we analyzed if BPA could have disrupting effects on the molecules highlighted in the pathway. To this end, we first checked the most suitable BPA concentration for inducing gene expression by testing the induction of the estrogen

Figure 4

Lipid accumulation. (A) Mature adipocytes untreated or treated with 1, 10, or 100 nM BPA for 24 h stained with oil red O ($\times 400$ magnification). (B) Mean lipid area per cell after ORO staining. (C) Cellular triglyceride content. E₂ treatment (1 nM) was used to compare BPA effects. Data are

expressed as means $\pm$ s.e.m. of three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ as compared to untreated cells. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JME-14-0282>.

responsive receptors ER α , ER β , and ERR γ , which are known to be expressed in human adipose tissue and to interact with BPA. Low doses of BPA modulated the abundance of the ER α transcript, whereas no changes in ER β mRNA levels were found. This was not surprising, given that BPA activity varies depending on specific estrogen receptor expression and on the distribution of those receptors within tissues. Specifically, it has been reported that in adipose tissue, ER β is less expressed than ER α (Hugo *et al.* 2008).

Overexpression of ERR γ confirms the high binding affinity of BPA for ERR γ (Takayanagi *et al.* 2006, Okada *et al.* 2008). ERR γ , a constitutive activator of transcription, is able to modulate the estrogen-signaling pathway not by binding directly to E₂, but by controlling

the transcription of essential genes that regulate metabolic processes (Liu *et al.* 2007, Giguere 2008).

The fact that ERR γ expression also increased after E₂ treatment must not be considered misleading. Indeed, similar expression does not indicate that this corresponds to similar mechanisms; instead, it suggests that BPA may exert its action through non-classical estrogen receptors, which results in independent effects (Hugo *et al.* 2008, Tohme *et al.* 2014). The high BPA accumulation in adipose tissue also suggests that it acts as an endocrine disruptor by increasing the abundance of ERR γ transcripts while also affecting the regulation of metabolic pathways.

More interesting is the modulation of GP30 expression, because GP30 reflects how BPA acts through the non-canonical estrogenic pathway. GPR30 is a seven-

Figure 5

BPA affects active insulin secretion. (A) Active insulin secreted in culture medium after BPA treatment of PANC-1 cells. Data are expressed as means $\pm$ s.e.m. of three independent experiments. (B) *PCSK1* mRNA expression in PANC-1 cells. Data are expressed as means $\pm$ s.d. of three independent experiments. * $P < 0.05$ as compared to the control.

transmembrane estrogen receptor that increases the activity of adenylate cyclase as a second messenger. The subsequent increase in intracellular cAMP regulates phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) signaling, a well-known regulator of glucose metabolism. BPA has been reported to elicit rapid responses by binding GPR30 and activating alternative non-genomic estrogen signaling pathways (Thomas & Dong 2006). In particular, the down-regulation obtained with 10 nM BPA is a novelty and leads us to hypothesize that BPA could influence PI3K/Akt signaling. The data of this study agrees well with previous studies that show that GPR30 knockout mice displayed impaired glucose tolerance, reduced body growth, and increased body weight (Martensson *et al.* 2009, Ford *et al.* 2011). Our data can be considered additional evidence that BPA at low doses may act not only via ER α but also by activating non-canonical ER pathways.

The up-regulation of leptin mRNA levels is very intriguing and agrees with the results of a previous study (Angle *et al.* 2013). Leptin production by adipocytes is proportional to their fat content; leptin serum levels are higher in obese subjects, and are directly correlated to adipose mass and adipocyte contents (Fried *et al.* 2000, Yang & Barouch 2007). These considerations clearly indicate that BPA treatment may have an obesogenic effect.

The BPA doses used in this study showed that the well-established non-monotonic dose response related to the BPA action, which regulates endocrine pathways, exhibits a U or an inverted U shape (Vom Saal *et al.* 2012, Boucher *et al.* 2014, Liang *et al.* 2014, Vandenberg 2014). This suggests that BPA concentrations in the typical range of environmental human exposure induce non-linear effects during childhood.

IPA pathway analysis allowed us to identify the altered expression of the genes involved in adipose tissue inflammation as well as some key genes involved in obesity and metabolic diseases.

CCL20 encodes for a cytokine involved in immunoregulatory and inflammatory processes as well in metabolism. Elevated serum CCL13 and CCL20 concentrations are found in overweight subjects during chronic inflammation. Furthermore, *CCL20* is directly correlated with BMI (Hashimoto *et al.* 2006, Duffaut *et al.* 2009, Villaret *et al.* 2010).

IL18 and *IL1B* are important regulators of inflammatory responses. As pro-inflammatory factors that are also secreted from adipocytes, interleukins contribute to the chronic low-grade inflammatory state of adipose depots that is often observed in obesity and type 2 diabetes (Samaras *et al.* 2010, Banerjee & Saxena 2012). Their expression and secretion in adipose tissue increase in metabolic disorders (Esser *et al.* 2013). Their increased production could also explain the link between obesity and metabolic diseases (Samaras *et al.* 2010, Ouchi *et al.* 2011).

CD36 is an integral membrane protein that binds oxidized lipoproteins and lipids (Endemann *et al.* 1993). It plays a key role in fatty acid and glucose metabolism, and its dysregulation is involved in glucose intolerance and diabetes (Hajri *et al.* 2002, Rac *et al.* 2007).

Similarly, *FABP4* is produced in adipocytes, and it is involved in fatty acid uptake and metabolism; it is also an important contributor to metabolic dysfunction in obesity-induced chronic inflammation and dyslipidemia (Hardaway & Podgorski 2013).

PCSK1 encodes pro-protein convertase subtilisin/kexin type 1 (PC1/3), a pro-insulin-processing enzyme that regulates insulin biosynthesis. PC1/3 cleaves pro-insulin to produce active insulin. Mutations of or deficiencies in PC1/3 are thought to cause early obesity (Creemers *et al.* 2012, Turpeinen *et al.* 2013).

To investigate whether BPA can mimic estrogen activity or antagonize E₂ function, we first analyzed the expression of the genes by qRT-PCR. The results showed that most of these genes, which were previously associated with metabolic disorders, had opposite expressions in the two treatments. Adipose tissue represents the major source of the pro-inflammatory cytokines and chemokines observed in obesity, and chronic low-grade inflammation is generally associated with obesity. It is known that CD36 null mice display enhanced insulin responsiveness, which is associated with a reduction in fat deposition (Hajri *et al.* 2002). *IL1B* has recently been associated with insulin resistance and type 2 diabetes (Tack *et al.* 2012), and its

inhibition reduces hyperglycemic inflammation in obese mice (Owyang *et al.* 2010).

The expression of FABP4 and PCSK1 also suggests an obesogenic role for BPA, because elevated FABP4 plasma levels are associated with metabolic syndrome (Xu *et al.* 2007), whereas a deficiency of PCSK1 leads to serious multi-hormonal disorder marked by early-onset obesity (Jackson *et al.* 1997).

To assess the disrupting effects of BPA, we investigated the physiological outcomes of BPA treatment for all genes for which the microarray expression was confirmed by qRT-PCR, which had opposite expression from that seen in the E₂ and BPA treatments.

The induction of inflammation was assessed by measuring IL1B, IL18, and CCL20 secretion. BPA-treated mature adipocytes show increased secretion of all of these chemokines (Fig. 3). In agreement with the recent finding that BPA up-regulates inflammatory pathways in 3T3-L1 cells and in adult human subcutaneous adipocytes (Valentino *et al.* 2013), our data corroborated the hypothesis that the strong relationship between BPA and the inflammatory state of adipose depots that occur in late obesity and in metabolic syndrome can be induced during childhood.

Inflammation and lipid metabolism are closely associated (Iyer *et al.* 2010), so we tested whether up-regulation of CD36 and FABP4 induced adipose depot expansion and triglyceride accumulation. The results showed a significant increase in intracellular lipid droplets and in triglyceride concentration. Up-regulation of *FABP4* and *CD36* indicated that BPA affects adipocyte lipid storage, which leads to cellular hypertrophy. A possible consequence could be a metabolic dysregulation that results in a resistin secretion increase as well as in a decrease in adiponectin production *in vitro* (C Menale, A Grandone, C Nicolucci, G Cirillo, S Crispi, A Di Sessa, S Rossi, D G Mita, L Perrone, E M Del Giudice, N Diano, unpublished observations). In addition, this up-regulation might impair glucose metabolism, as confirmed by preliminary experiments performed in a mouse model (C Menale, personal communication). This result led us to hypothesize that BPA might increase the oxidative stress of hypertrophic adipocytes and might contribute to the inflammation and dysregulation of free fatty acid efflux (Zha & Zhou 2012).

Finally, we tested whether *PCSK1* down-regulation by BPA affects active insulin secretion in the human PANC-1 cell line. The results showed that BPA effectively impairs active insulin production and that its decrease is related to *PCSK1* mRNA down-regulation. This novel view regarding BPA activity indicates that PCSK1 could be considered a new player that is involved in the endocrine disruption

that is caused by BPA, because it participates in metabolic disorders in childhood by playing a key role in the deregulation of insulin biosynthesis, which in turn, considering the physiological context, could affect the pancreas–adipose tissue crosstalk. *In vivo* preliminary observations by C Menale (personal communication) indicate that BPA exposure in the mouse impairs glucose tolerance by compromising insulin production and/or secretion. The putative key role of *PCSK1* dysregulation needs to be further investigated *in vivo*.

Taken together, our results suggest that the modulation of gene expression by BPA leads to fatty acid metabolism dysfunction, pro-inflammatory cytokine production, and insulin secretion; all of these are conditions that can be ascribed to metabolic disorders.

In conclusion, this study shows that BPA at environmental doses in mature adipocytes from children acts by modulating gene expression, which leads to molecular changes that promote metabolic disorders. Considering that the persistence of BPA in the environment and in the human body, specifically in adipose tissue, might be an important risk factor, the results of this study provide a strong argument for further research *in vitro* and *in vivo* on the contribution of BPA exposure to the development of obesity and diabetes.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JME-14-0282>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

S C, C M, D G M, and N D developed the conceptual framework for the study and coordinated the data discussion; S C and C M conceived and designed the experiments and wrote the paper; C M, M T P, and G C performed the experiments; R A C analyzed the microarray data; C M, L M, and E M D G analyzed the data; A P provided the subcutaneous adipose tissue.

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