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Low-dose Bisphenol-A regulates inflammatory cytokines through GPR30 in mammary adipose cells

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Abstract

The dramatic rise in obesity and metabolic syndrome can be related, at least in part, to environmental chemical factors such as Bisphenol-A (BPA). In this study, we aimed to understand the effects of low-dose Bisphenol-A on the human mature adipocytes and stromal vascular fraction (SVF) cells, obtained from subcutaneous mammary adipose tissue of overweight female patients, undergoing surgical mammary reduction. 24 and/or 48-h exposure to BPA 0.1 nM elicited significant increase of the inflammatory molecules interleukin-6 (IL-6), interleukin-8 (IL-8), monocyte chemo-attractant protein 1α (MCP1α) and induced G protein-coupled estrogen receptor 30 (GPR30) levels more than two-fold both in mature adipocytes and SVF cells. These effects were similar to that obtained in the presence of GPR30-specific agonist G1 (100 nM) and were reverted by G15 (1 μM), a GPR30-selective antagonist. As a result of BPA-GPR30 signaling activation, fatty acid synthase (FAS) and leptin mRNA levels were significantly higher upon BPA exposure ($P < 0.05$) in mature adipocytes, with an opposite effect on adiponectin (ADIPOQ). In addition, an increase in SVF cell proliferation and ERK1/2 phosphorylation, was observed, compared to untreated cells. G15 reverted all of these effects. Interestingly, the action of BPA on SVF cell growth was mimicked by IL-8 treatment and was reverted by incubation with anti-IL8 antibodies. All these data suggest that BPA at 0.1 nM, a ten times lower concentration than environmental exposure, increases the expression of pro-inflammatory cytokines via GPR30 both in mature mammary adipocytes and in SVF cells with a possible involvement of IL-8.

Key Words

- Bisphenol-A
- Adipocytes
- SVF cells
- GPR30
- Inflammatory cytokines

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Introduction

The role of environmental chemical factors with endocrine-disrupting activities is gaining increasing evidence. The ability of environmental estrogen-like molecules to bind classical and non-classical estrogen receptors (ERs) has been well documented (Thomas & Dong 2006). Interestingly, these endocrine disruptors can activate multiple intracellular rapid signaling pathways, which in turn, induce the release of other extracellular signaling systems, possibly leading to detrimental effects on human health (Acconcia *et al.* 2015, Nadal *et al.* 2017). On the other hand, the role of estrogen in control of energy balance, in adipose tissue (AT) metabolism, in glucose homeostasis regulation and in fat distribution is well documented (Mauvais-Jarvis *et al.* 2013, Kim *et al.* 2014, Wang *et al.* 2016). Among endocrine disruptors with estrogen-like activity, Bisphenol-A (BPA), a monomer of polycarbonate plastics with lipophilic features, at very low doses contributes to alter metabolic homeostasis, affecting glucose and lipid metabolism in AT, through activation of inflammatory pathways (Valentino *et al.* 2013, Savastano *et al.* 2015). Since BPA, although in a very small amount, can migrate from plastic to food and drink, mainly due to hydrolysis induced by heat, acid or basic conditions, the chronic ingestion and exposure by humans is responsible for its detectable levels in plasma, urine and breast milk (Vandenberg *et al.* 2007). BPA may have deleterious effects on different tissues and systems. In particular, our previous *in vitro* studies, have reported the role of BPA in activation of inflammatory pathways, at doses consistent with environmental exposure (Ariemma *et al.* 2016, Camarca *et al.* 2016). The mechanisms by which BPA may act are still enigmatic and several speculations have been proposed. One hypothesis is related to the BPA's ability to bind classical and non-classical ER. Interestingly, when BPA binds classical estrogen receptor at nanomolar doses, it exerts a stronger estrogen-like activity than at micromolar doses (Nadal *et al.* 2000, Wetherill *et al.* 2007, Angle *et al.* 2013). Recently, a large body of data has evidenced that BPA's deleterious effects can be mediated by binding non-classical ER, such as GPR30 (Thomas & Dong 2006, Alonso-Magdalena *et al.* 2012, Sheng *et al.* 2013). *GPR30* gene was first described in breast cancer (Carmeci *et al.* 1997) and subsequently detected in a wide range of tissues, although the consequences of inappropriate activation of this estrogen-initiated signaling pathway remain to be elucidated (Belcher 2008). In fact, GPR30 is reported to mediate estrogen-dependent rapid signaling events as well as transcriptional activation, independent of classical estrogen nuclear receptors. The consequences of its activation in different cell types, particularly in tumor cells, are the regulation of cell growth, migration, and apoptotic cell death. BPA, as environmental lipophilic estrogen, exhibits the ability to bind to GPR30 and to activate transduction pathways, eliciting a rapid intracellular response both in normal and neoplastic cells (Thomas & Dong 2006, Bouskine *et al.* 2009, Sheng & Zhu 2011, Chevalier *et al.* 2012, Sheng *et al.* 2013, Sharma *et al.* 2018). Notably, these effects occur through calcium mobilization, upregulation of ERK1/2, phosphatidylinositol 3-kinase (PI3K) and second messenger adenyl cyclase (cAMP), mainly via transactivation of epidermal growth factor receptors (EGFRs) (Filardo & Thomas 2005, Revankar *et al.* 2005, Thomas & Dong 2006, Albanito *et al.* 2008). In addition, the BPA non-genomic effects are evidenced in mouse endothelial cells, as well as in rat distal colonic epithelium (Doolan & Harvey 2003) or hippocampal neurons (Tanabe *et al.* 2006), with stimulation of nitric oxide (NO) synthesis (Noguchi *et al.* 2002). Thus, the non-genomic estrogen actions can be the basis for the activation of alternative mechanisms of BPA, leading to different biological effects in a broad range of tissues and cell lines.

In the present work, we have investigated the consequences of low-dose BPA on cultured mature adipocytes and stromal vascular fraction (SVF) cells isolated from human mammary AT biopsies of overweight female patients, undergoing surgical mammary reduction. Our results reveal that 0.1 nM BPA regulates the production of inflammatory cytokines through the activation of GPR30, suggesting this molecule as a potential therapeutic target against the BPA-induced damages in AT.

Materials and methods

Reagents

BPA was a generous gift of Prof. C. Crescenzi (Department of Pharmaceutical and Biomedical Science, University of Salerno, Fisciano - SA, Italy) and was dissolved in ethanol at 100 nM concentration and further diluted in the media to obtain 0.1 nM concentration. For Western blot analysis, antibodies against ERK1/2 (Cat# sc-514302) and 14.3.3 (Cat# sc-7681) were purchased from Santa Cruz Biotechnology. Phospho-Thr202/Tyr204ERK (Cat# 9101) antibody was obtained from Cell Signaling Technology. GPR30 antibody (Cat# ab39742) was purchased from Abcam. Protein electrophoresis and real-time PCR reagents were from Bio-Rad. Western blotting and ECL

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reagents were purchased from Amersham Biosciences (Arlington Heights, IL, USA). The compounds G1-specific GPR30 agonist (100 nM) (Cat# 3577) and G15-specific GPR30 antagonist (1 μM) (Cat# 3678) were from Tocris Bioscience. Human recombinant IL-8 (Cat# 200-08), and anti-IL-8 blocking antibody (Cat. #500-P28) were purchased from PeproTech (London, UK). Monoclonal antibodies against human CCL5 were from Abcam.

All the other chemicals were from Sigma-Aldrich. Media and antibiotics for cell culture were from Lonza (Lonza Group Ltd, Basel, Switzerland), sera from Gibco.

Cell culture and proliferation assays

Human AT samples were obtained from mammary subcutaneous AT biopsies of patients ($n=6$) undergoing surgical mammary reduction. Inclusion criteria were female sex, BMI range 26–28 kg/m^2, age range 35–50 years. Exclusion criteria were endocrine, metabolic, inflammatory or proliferative diseases, infections, alcohol or drugs, assumption. None of the enrolled women used anti-inflammatory agents and/or glucocorticoids in the last week.

The study was approved by the Ethic Committee of the University of Napoli 'Federico II' (Prot n. 138/16). The written informed consent was obtained before the bioptical procedure. The study protocol was conducted in accordance to the principles of the Declaration of Helsinki as revised in 2000.

AT was digested with collagenase and mature adipocytes and adipose-derived SVF cells were isolated and cultured as previously reported (Isakson *et al*. 2009).

For cell proliferation SVF cells were seeded in six-well culture plates in complete medium. SVF count was performed either by Burker chamber and with the TC10 Automated Cell Counter (Bio-Rad), according to the manufacturer's protocol. Both mature adipocytes and SVF cells were cultured in Dulbecco's modified Eagle's medium (DMEM): F12 supplemented with 10% fetal bovine serum (FBS) (1:1) and 2% glutamine, 100 IU/mL penicillin and 100 IU/mL streptomycin. Cultures were maintained in humidified atmosphere of 95% air and 5% CO_2 at 37°C. For all experiments, cells between passages 2 and 7 were used.

Western Blot analysis

Total cell lysates were obtained and separated by SDS-PAGE. Briefly, untreated cells (CTR) and cells incubated with 0.1 nM BPA for the indicated times, were solubilized for 20 min at 4°C with lysis buffer containing 50 mM HEPES, 150 mM NaCl, 10 mM EDTA, 10 mM Na4P2O7, 2 mM sodium orthovanadate, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin, pH 7.4, and 1% (v/v) Triton X-100. Lysates were clarified by centrifugation at 12,000 ***g*** for 20 min at 4°C. The protein concentrations in the cell lysates were measured using a Bio-Rad DC (detergent compatible) assay. Western blot analysis was performed as previously described (Cimmino *et al*. 2017).

PCR analysis

Two microliters of cDNA was used for the PCR performed in 25 μL consisting of 5× WonderTaq Reaction Buffer, 0.4 μM of forward and reverse primer (Table 1) and 0.05 units/μL of WonderTaq polymerase (Euroclone, Milan, Italy).The PCR was performed as follows: the WonderTaq enzyme was activated at 95°C for 15 min followed by 40 cycles of a three-step protocol (95°C for 1 min, 60°C for 15 s and 72°C for10 min) and a final elongation step at 72°C for 10 min. The reaction was visualized on a 1% agarose gel electrophoresis in TBE buffer 1×. Gels were stained with ethidium bromide and photographed under UV light at 305 nm. The PCR assays were performed at least twice.

Real-time RT-PCR analysis

Total cellular RNA was isolated at the indicated times from untreated (CTR) or incubated with 0.1 nM BPA SVF cells and mature adipocytes by using the Rneasy Kit (Qiagen) according to the manufacturer's instruction. 1 μg of cell RNA was reverse transcribed using Super-Script III Reverse Transcriptase (Life Technologies). PCR reactions were analyzed using IQTM SYBR Green Supermix (Bio-Rad). Reactions were performed using Platinum SYBR Green qPCR Super-UDG using an iCycler IQ multicolor Real-Time PCR Detection System (Bio-Rad, CA). All reactions were performed in triplicate and peptidylprolyl isomerase A (PPIA) was used as an internal standard. Primer sequences used are described in Table 1.

Statistical analysis

All analyses were performed using GraphPad Prism 7.0 software. A non-parametric Mann–Whitney test was applied to compare two groups, while Kruskal–Wallis with Dunn's post-test analysis was used to determine differences between more groups. P value<0.05 was considered statistically significant. All values were

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Table 1 Primer sequences used in human mature adipocytes and SVF cells.

Primers	Gencode gene	Sequences
ERα	ENSG00000091831.23	Forward: 5′-AGTGGGAATGATGAAAGGTG-3′ Reverse: 5′-CAGTAAGCCCATCATCGAAG-3′
ERβ	ENSG00000140009.18	Forward: 5′-CAGCTGCTGGCTTTTTGGAC-3′ Reverse: 5′-CTCTTCGCCCTGCAAGTTTC-3′
GPR30	ENSG00000164850.14	Forward: 5′-CTTCATCGTGCCCTTCGCC-3′ Reverse: 5′-GAGAAGGCGGCGAGGTTGA-3′
FAS	ENSG00000169710.8	Forward 5′-GAGAGCCTCTTCTCCAGGGT-3′ Reverse 5′-CTTCA GAGACTCCACCCAGC-3′
ADIPOQ	ENSG00000181092.9	Forward : 5′-ACTGCAGTCTGTGGTTCTGA-3′ Reverse : 5′-TCGTGGTTTCCTGGTCATGA-3′
LEPTIN	ENSG00000174697.4	Forward 5′-CACACACGCAGTCAGTCTC-3′ Reverse 5′-GAGGTTCTCCAGGTCGTTG-3′
IL-8	ENSG00000169429.10	Forward: 5′-TTCCAAGCTGGCCGTGGCTC-3′ Reverse: 5′-TGTGTTGGCGCAGTGTGGTCC-3′
IL-6	ENSG00000136244.11	Forward: 5′-TTCCAAAGATGTAGCCGCCC-3′ Reverse: 5′-ACCAGGCAAGTCTCCTCATT-3′
MCP1α	ENSG00000108691.9	Forward: 5′-ACCTGGACAAGCAAACCCAA-3′ Reverse: 5′-AGGGTGTCTGGGGAAAGCTA-3′
PPIA	ENSG00000196262.13	Forward: 5′-TACGGGTCCTGGCATCTTGT-3′ Reverse: 5′-GGTGATCTTCTTGCTGGTCT-3′

expressed as median and interquartile range. The number of four repetitions for each experiment was established in advance of analyzing the data.

Results

Low-dose BPA effect on inflammatory markers in mammary mature adipocytes and in SVF cells

Mature adipocytes and SVF cells, isolated from human AT biopsies ($n=6$), were cultured for 24 h in the presence of 0.1 nM BPA. This concentration was not toxic for the cells (data not shown). Hence a panel of cytokines was analyzed. BPA increased IL-8, MCP1α and IL-6 by 13.3-, 2.3-, and 12.7-fold in mature adipocytes (Fig. 1A) and by 1.4-, 1.5-, and 1.4-fold in SVF cells. In parallel, also TNFα and IL-1β were induced by BPA, while IL-10, which acts generally as an anti-inflammatory molecule, had an opposite trend (Supplementary Fig. 1, see section on supplementary data given at the end of this article). Similar results were observed after incubating SVF cells with BPA for 48 h (Fig. 1B, Supplementary Fig. 1).

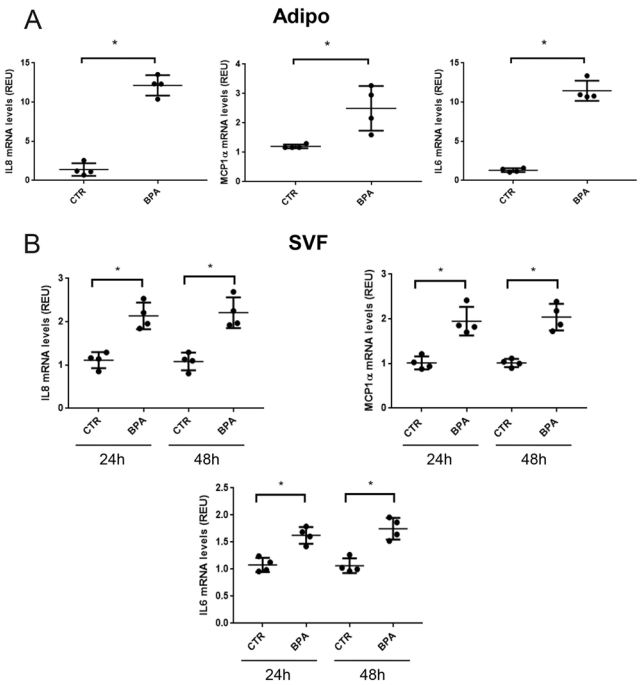

Figure 1
Role of low-dose Bisphenol-A (BPA) on inflammation in mature mammary adipocytes and in SVF cells. Mature adipocytes (A) and SVF cells (B), isolated from mammary adipose tissue biopsies ($n = 6$), were grown in suspension and treated with 0.1 nM BPA for 24 h and 48 h. IL-8, MCP1α and IL-6 mRNA levels were analyzed by real-time RT-PCR analysis, expressed as relative expression unit (REU). Data were normalized by the amount of PPIA mRNA, used as internal control. Bars represent the median and interquartile range of four independent experiments, each performed in triplicate. Asterisks denote statistically differences (*$P < 0.05$) upon BPA treatment compared to control cells (CTR).

Low-dose BPA effect on GPR30 expression in mammary mature adipocytes and in SVF cells

BPA may exert its effects through the binding to classical/non-classical estrogen receptor. In mature adipocytes, basal levels of both ERα and ERβ were almost undetectable, while, GPR30 mRNA levels were clearly evident (Fig. 2A). Moreover, 0.1 nM BPA exposure significantly increased

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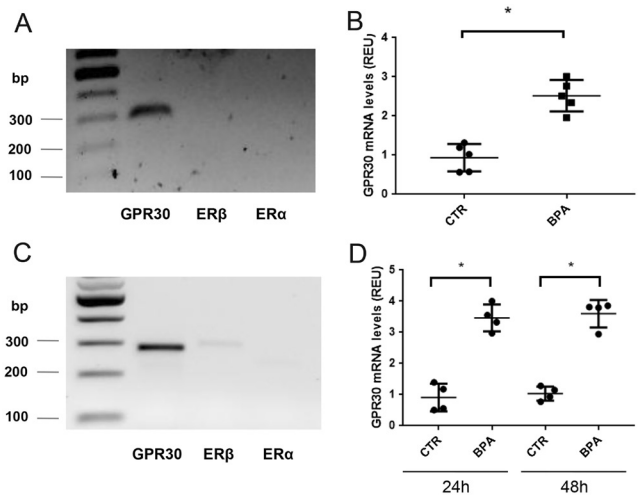

Figure 2
BPA activates non-classical GPR30 receptor in mammary adipocytes and in SVF cells. (A and C) PCR assays was conduct to determine the presence of several receptors in mature mammary adipocytes and in SVF cells. The reaction was visualized on a 1% agarose gel electrophoresis in TBE buffer 1×. Gels were stained with ethidium bromide and photographed under UV light at 305 nm. MW represents a 100-bp size marker generated by GeneRuler 100 bp Plus DNA ladder. The PCR assays were performed at least twice. (B and D) GPR30 mRNA levels were analyzed by real-time RT-PCR analysis expressed as relative expression unit (REU). PPIA was used as loading control. Bars represent the median and interquartile range of four independent experiments, each performed in triplicate. Asterisks denote statistically differences (*$P < 0.05$) upon BPA treatment compared to control cells (CTR).

GPR30 levels (Fig. 2B), without affecting ERα and ERβ expression (data not shown). Similar as in mature adipocytes, in SVF cells, a very faint signal was detectable for ERα and ERβ, while GPR30 was much higher (Fig. 2C). A 2.3-fold increase in GPR30 expression was observed after 24 and 48 h of incubation with 0.1 nM BPA ($P<0.05$) in SVF cells (Fig. 2D). To assess BPA ability to trigger signals via GPR30, we treated mature adipocytes with GPR30 selective agonist/antagonist for 24 h. As shown in Fig. 3A, G1 agonist (100 nM) effect on GPR30 mRNA levels was comparable to BPA, while G15 (1 µM) inhibited BPA-induced enhancement of GPR30. Similar results were also obtained for protein levels (Fig. 3B).

IL-8, IL-6 and TNFα levels were significantly increased by BPA, while adiponectin decreased. MCP1α, leptin, IL-1β and IL-10 did not statistically change (Fig. 3C, D, E, F and G, Supplementary Fig. 1). In parallel, IL-8, MCP1α, IL-6 and leptin levels were significantly induced upon incubation with G1, with an opposite effect on adiponectin (albeit difference did not reach statistical significance) (Fig. 3C, D, E, F and G). BPA-induced modifications of MCP1α, adiponectin, TNFα and IL-1β were prevented by G15 (Fig. 3D and G, Supplementary Fig. 1).

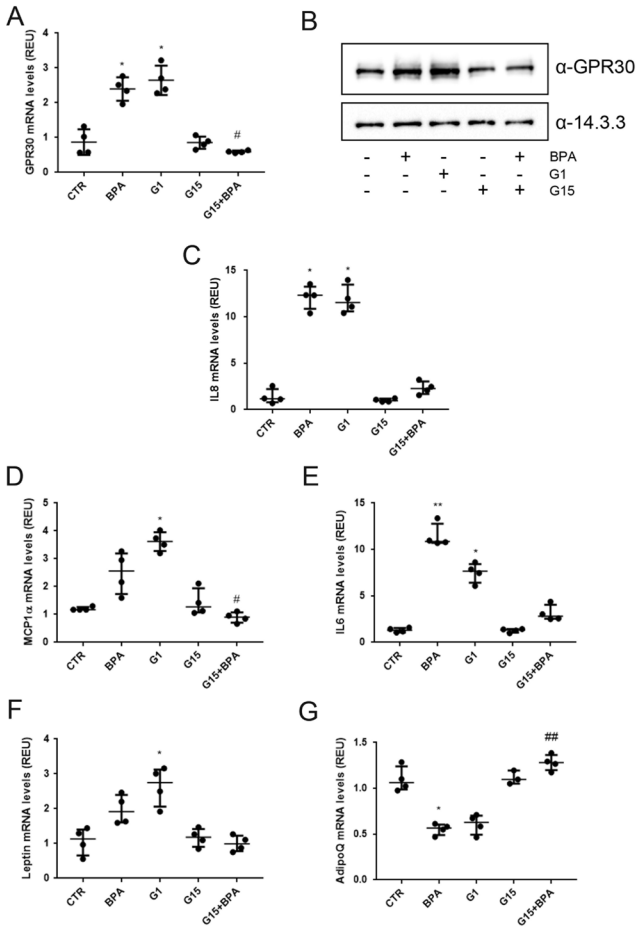

Figure 3
BPA effect on inflammatory markers involves GPR30 expression in mature mammary adipocytes. GPR30 (A), IL-8 (C), MCP1α (D), IL-6 (E), leptin (F) and adiponectin (G) mRNA levels were assayed on isolated mature adipocytes from mammary adipose tissue biopsies ($n = 6$) after 24 h upon 0.1 nM BPA incubation and in presence of G1 (100 nM) and G15 (1 µM) by real-time RT-PCR analysis, expressed as relative expression unit (REU). Bars represent the median and interquartile range of four independent experiments. Asterisks indicate statistically significant differences (*$P < 0.05$; **$P < 0.01$) upon BPA and G1 treatment compared to non-treated cells (CTR); (#$P < 0.05$; ##$P < 0.01$) upon BPA 0.1 nM stimulated cells treated with G15 compared to cells treated with BPA. (B) Protein lysates from mature mammary adipocytes, untreated or treated with BPA at 0.1 nM, G1 at 100 nM and G15 at 1 µM, were analyzed by Western blot using antibodies for GPR30 and 14.3.3 used as loading control. The autoradiographs are representative of three independent experiments.

Very similar results were obtained by treating SVF cells with G1 and G15 for 24 h. Indeed, while G1 showed an almost comparable effect with BPA, G15 prevented BPA effect on GPR30 ($P<0.059$) and on the expression of IL-8, MCP1α, IL-6 and TNFα, while not affecting IL-1β and IL-10. These effects were also evident after 48 h treatment (Fig. 4, Supplementary Fig. 1). Concentration of G1 and G15 were not toxic for both cell types (data not shown).

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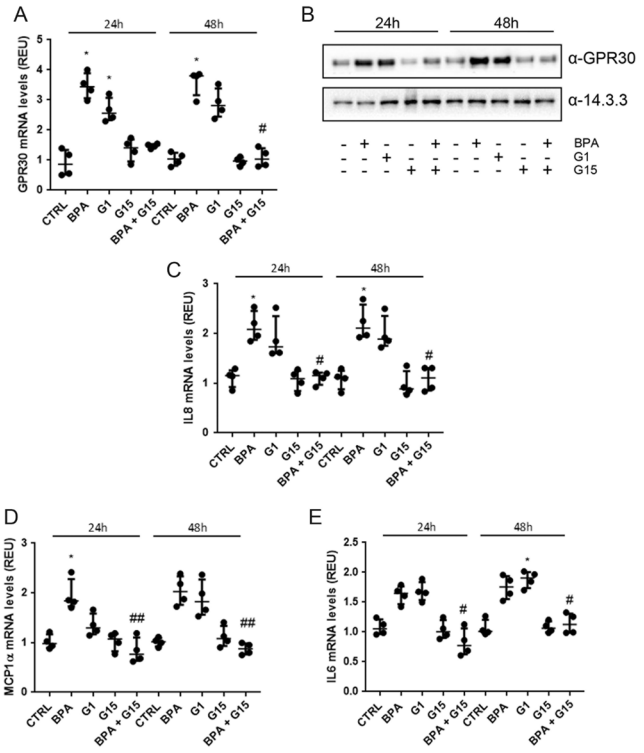

Figure 4
BPA effect on inflammation involves GPR30 expression in SVF cells. GPR30 (A), IL-8 (C), MCP1α (D), IL-6 (E) mRNA levels were assayed on SVF cells after 24 and 48 h upon 0.1 nM BPA incubation or without (CTR) and in presence of G1 (100 nM) and G15 (1 μM) by RT-real-time PCR analysis, expressed as relative expression unit (REU). Bars represent median and interquartile range of four independent experiments. Asterisks indicate statistically significant differences (*$P < 0.05$) upon BPA treatment compared to non-treated cells (CTR); (#$P < 0.05$; ##$P < 0.01$) upon BPA 0.1 nM stimulated cells treated with G15 compared to cells treated with BPA. (B) SVF cells were solubilized and protein samples analyzed by Western Blot with GPR30 and 14.3.3 antibody was used for normalization. Blots were revealed by ECL and autoradiograph is representative of three independent experiments.

Low-dose BPA-induced adipocyte FAS expression and SVF cell proliferation involves GPR30

In order to explain the possible downstream effects of BPA-GPR30 induction, we evaluated fatty acid synthase (FAS) mRNA levels in mammary mature adipocytes. Interestingly, a 3.5-fold increase of FAS mRNA was observed in the presence of BPA and G1, while G15 inhibited this effect (Fig. 5A). ERK1/2 phosphorylation, instead, was tested as a marker of GPR30 activation in SVF cells. BPA and G1 stimulation was paralleled by increased levels of ERK1/2 phosphorylation, with no major changes in ERK1/2 protein abundance (Fig. 5B and C). BPA effect on ERK1/2 phosphorylation was also blunted by G15, suggesting a relevant contribution of ERK1/2 activation in GPR30-mediated cellular effects (Fig. 5C). GPR30

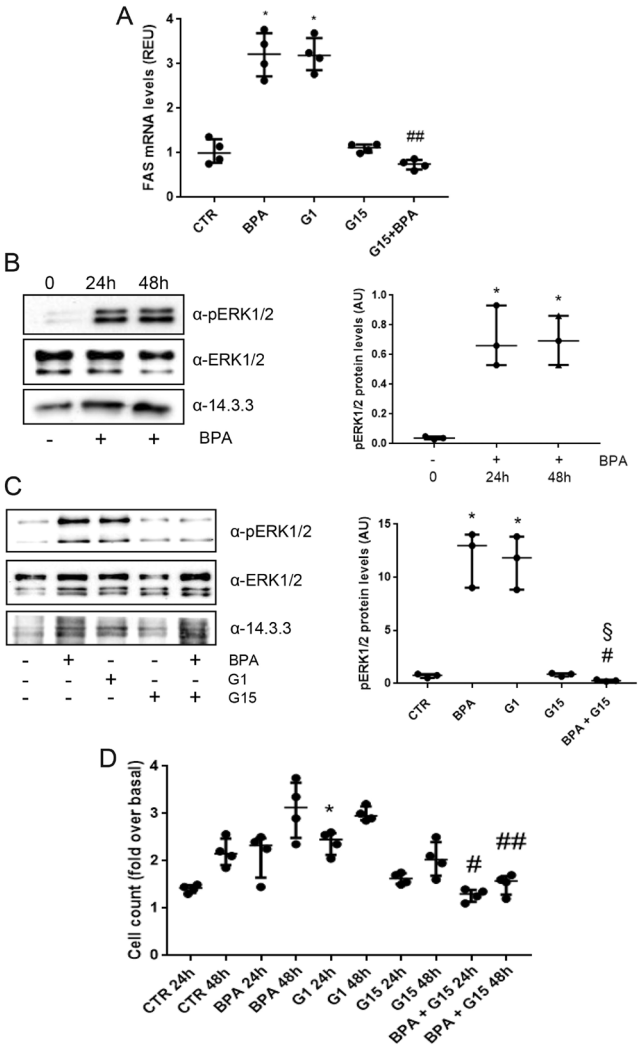

Figure 5
GPR30 involvement in BPA-mediated proliferation. FAS mRNA levels (A) were assayed by RT-Real-Time PCR, in mature mammary adipocytes untreated (CTR) or treated with 0.1 nM BPA for 24 h and in presence of G1 (100 nM) and G15 (1 μM). Protein lysates from SVF cells, untreated or treated with BPA at 0.1 nM (B) and treated with G1 at 100 nM and G15 at 1 μM (C), were analyzed by Western blot using antibodies for pERK1/2, ERK1/2, and 14.3.3 used as loading control. The autoradiographs are representative of three independent experiments. (D) SVF cells were grown in DMEM supplemented with 10% FBS. Then, cells were harvested, counted and expressed as fold over basal, after 24 and 48 h of 0.1 nM BPA treatment or untreated (CTR) and in presence of G1 and G15. Asterisks denote statistically significant differences (*$P < 0.05$) upon BPA treatment compared to non-treated cells (CTR); (#$P < 0.05$; ##$P < 0.01$) upon BPA 0.1 nM stimulated cells treated with G15 compared to cells treated with BPA (§$P < 0.05$) upon 0.1 nM BPA stimulated cells in combination with G15 compared to G15-treated cells.

activation may enhance cellular proliferation (Bouskine *et al.* 2009, Lin *et al.* 2009, Pandey *et al.* 2009). Indeed, SVF cells displayed an increased proliferative trend in presence of BPA upon 24 h and 48 h of incubation, respectively

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(Fig. 5D). Consistently, G15 reduced cell number in BPA-treated cells.

IL-8 is a downstream mediator of BPA-GPR30 signaling

Very recent data indicated IL-8 as a stimulator of SVF cell proliferation (Parsons *et al.* 2018). Hence, SVF cell proliferation was measured upon stimulation with either Bisphenol-A or IL-8 in the presence or in the absence of IL-8-blocking antibodies. Interestingly, inhibition of IL-8 reverted BPA effect on cell growth, suggesting that IL-8 may be a downstream effector of BPA action. At variance, CCL-5-blocking antibodies, used as negative control, had no effects on BPA activity (Fig. 6).

Discussion

In the last decades the dramatic increase of several metabolic diseases has been associated not only to the modification in lifestyle, as emphasized by the increased consumption of fat-rich food and sedentary activities, but also to some environmental chemicals, defined as endocrine disruptors, which include Bisphenol-A (BPA). BPA, which is present in many commercial products, including food and water containers, baby bottles beverage cans and linings of metal food, can migrate from these objects to the adult and fetal human plasma, urine, and breast milk, accumulating particularly in the AT (Hugo *et al.* 2008). Both *in vitro* and *in vivo* studies have

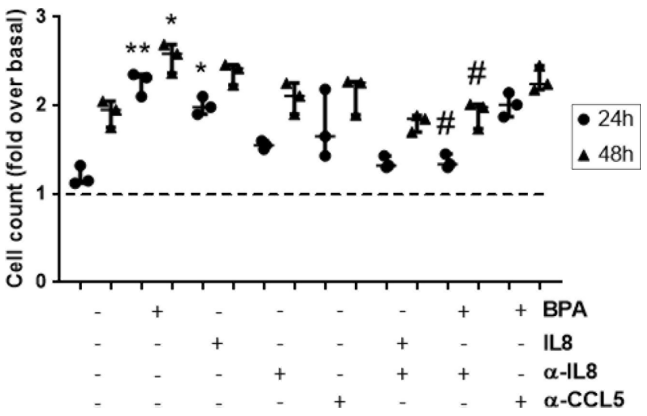

Figure 6
IL-8 increases cell proliferation via GPR30. SVF cells were grown in DMEM supplemented with 10% FBS. Then, cells were harvested, counted and expressed as fold over basal, after 24 and 48 h of incubation with BPA 0.1 nM or without (CTR) and in presence of IL8 (10 ng/mL), α-IL8 (1 ng/mL) and α-CCL5 (6 µg/mL). Asterisks denote statistically significant differences (*$P < 0.05$; **$P < 0.01$); (#$P < 0.05$) upon BPA 0.1 nM stimulated cells treated with G15 compared to cells treated with BPA.

reported adverse effects of BPA, as well as its association with inflammation, obesity and impaired metabolism also at nanomolar concentrations (Welshons *et al.* 2006, Hugo *et al.* 2008, Rubin 2011, Rochester 2013, Mirmira & Evans-Molina 2014, Valentino *et al.* 2016). We have previously shown that chronic exposure to 1 nM BPA induces hypertrophic and dysfunctional adipocytes (Ariemma *et al.* 2016) and its plasma levels are strictly associated to inflammatory markers, visceral obesity and insulin resistance (Savastano *et al.* 2015). In addition, Hugo *et al.* suggested that BPA action is not confined only to one AT type, but impairs adiponectin release from breast adipose explants obtained from women undergoing breast reduction, from abdominal subcutaneous fat from subjects undergoing abdominoplasty and from visceral and subcutaneous AT from morbidly obese individuals undergoing gastric bypass surgery (Hugo *et al.* 2008). However, since the potential toxic dose of Bisphenol-A is still not clear, in this study we have evaluated the effects of a lower dose of BPA (0.1 nM) and the possible molecular mechanisms of its action on both mature mammary adipocytes and SVF cells. We focused our attention on two different cell types as isolated mature adipocytes maintain their viability only for a short term. Conversely, SVF cells are stable over long-term culture, easily proliferate *in vitro*, and possess the potential to create diverse lineages of cells (Liu *et al.* 2017). Moreover, several studies suggest that part of the deleterious effects mediated by BPA exposure happens during the embryonic critical window, then, when tissues and organs are not completely developed (Vaiserman 2014, Alonso-Magdalena *et al.* 2015*a*, *b*). Incubation of both cell types with 0.1 nM BPA increases the expression of specific inflammatory and metabolic markers including IL-8, MCP1α, IL-6 and leptin, with an opposite effect on the adiponectin.

BPA exerts its xenoestrogenic activity by binding the classical nuclear estrogen receptors (ER) α and ß (Acconcia *et al.* 2015). However, a novel transmembrane estrogen receptor, known as G protein-coupled estrogen receptor (GPR30), can be activated by Bisphenol-A in male reproductive system, regulating fertility and spermatogenesis, as well as in mammary gland (Wang *et al.* 2017, Perrot-Applanat *et al.* 2018). In this study we provided evidence that low-dose BPA increases the expression of GPR30 both in mature mammary adipocytes and in SVF cells, without affecting ERα and ERß. These data have been confirmed in presence of the GPR30-selective agonist G1 which has similar effect of BPA. Consistently, BPA effects are reverted by the GPR30-selective antagonist G15. Interestingly, our results are consistent with report

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by Dong *et al.* indicating that Bisphenol-A acts, at least in part, by increasing the levels of GPR30 (Dong *et al.* 2011). Other studies have suggested that BPA, due to its lipophilic characteristic and conformation, preferentially binds GPR30 (Blair *et al.* 2000, Matthews *et al.* 2000, Mueller *et al.* 2003, Thomas & Dong 2006).

Although we have not directly evaluated the activation of GPR30 by BPA, we have observed an increase of ERK1/2 phosphorylation and FAS expression, which can be considered as markers of a downstream GPR30 activation in SVF cells and in mature adipocytes, respectively (Dong *et al.* 2011, Wang *et al.* 2016). Indeed, isolated mature adipocytes maintain their viability only for a short time and, thus, SVF cells may represent a suitable model to study developmental action of the BPA-GPR30 signaling in the AT (Wang *et al.* 2012). On the other hand, human FAS is distributed mainly in cells with high lipid metabolism such as adipocytes (Kusakabe *et al.* 2000) and can be activated by GPR30 (Santolla *et al.* 2012). Interestingly, in parallel with the ERK1/2 phosphorylation, SVF cells show an increased proliferative trend in the presence of BPA. This result is very intriguing as it can be interpreted as a possible mechanism to offer more pre-adipocytes and, thus, to compensate the induced inflammatory state.

Among the large number of cytokines, IL-8 has been associated with a chronic inflammatory process in subjects with severe obesity, type 2 diabetes, atherosclerosis, cardiovascular disease and cancer (Straczkowski *et al.* 2002, Apostolakis *et al.* 2009, Qazi *et al.* 2011, Cimini *et al.* 2017). IL-8 may be upregulated by Bisphenol-A in several cell types, including cardiomyocytes and macrophages, and by G1 in murine model (Klint *et al.* 2017, Chen *et al.* 2018). Interestingly, BPA-induced SVF cell proliferation is markedly reduced in presence of IL-8 blocking antibodies, suggesting a role of this cytokine in the BPA action. Several authors have suggested a potential role of IL-8 in the impairment of AT metabolism. In particular, Kobashi *et al.* indicate that IL-8 can induce insulin resistance in adipocytes and hypothesize that the attenuation of IL-8 action might be a target for prevention of diabetes and its complications (Gerhardt *et al.* 2001, Kobashi *et al.* 2009). However, we cannot exclude that other molecules, including IL-6, MCP1α, can contribute to the BPA-GPR30 signaling in the AT and more studies need to be done to better clarify this point.

Thus, it could be speculated that BPA activates GPR30 which, in turn, induces cytokines secretion by adipocytes, at a much higher extent compared to their SVF precursors. Among these, IL-8 may contribute both to AT inflammation and to expansion of adipose mass (Fig. 7).

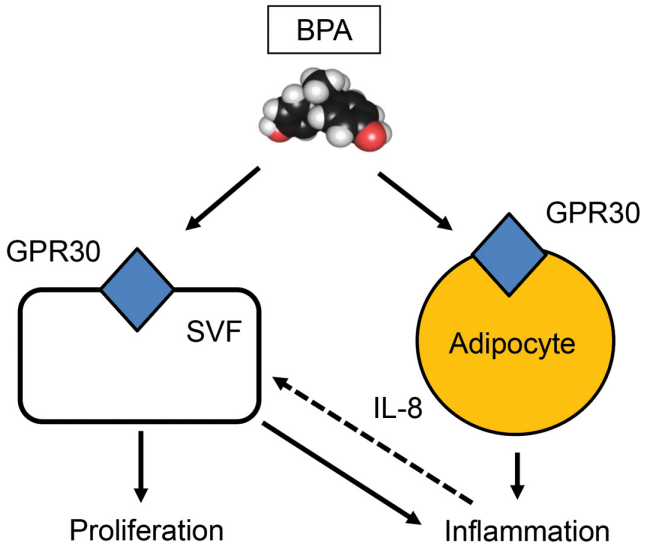

Figure 7
Schematic representation of low-dose BPA effect on GPR30 signaling. Low-dose BPA activates GPR30 estrogen receptor and induces pro-inflammatory cytokine production from mammary adipocytes and, to a lesser extent, from SVF cells. Among these, IL-8 may contribute to SVF proliferation and to the expansion of adipose mass.

In conclusion, the present study provides for the first time evidences that in cultured mature mammary adipocytes and SVF cells isolated from human AT biopsies, low doses of BPA acts through the non-classical GPR30 receptor. Although additional studies are required to establish the role of BPA-GPR30-mediated signaling in other tissues and organs, these findings may aid to better understand the molecular mechanisms involved in the BPA-induced mammary AT alterations.

Supplementary data
This is linked to the online version of the paper at https://doi.org/10.1530/JME-18-0265.

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
IC and FO were the main contributors in terms of conception, design, acquisition and interpretation of data and in drafting the article.

VDE, DL, PL, MRA, SC, FDA mainly contributed in conceptual design and acquisition of data. PF and FB mainly contributed in terms of conceptual design, analysis, interpretation and discussion of the results. RV mainly contributed in terms of conceptual design, interpretation and discussion of the results and supervision of the overall work. All the authors critically revised the article and approved the final version.

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