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Jonathan G. Boucher, Adèle Boudreau, Shaimaa Ahmed,
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***In Vitro* Effects of Bisphenol A β -D-Glucuronide (BPA-G) on Adipogenesis in Human and Murine Preadipocytes**

Jonathan G. Boucher, Adèle Boudreau, Shaimaa Ahmed, and Ella Atlas

Environmental Health Science and Research Bureau, Health Canada, Ottawa, Canada

Address correspondence to Ella Atlas, Environmental Health Science and Research Bureau, Health Canada, 50 Colombine Driveway, Ottawa, Ontario, K1A 0K9, Canada. Telephone: 613-957-0207. E-mail: ella.atlas@hc-sc.gc.ca

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Abstract

Background: Exposure to common environmental substances, such as bisphenol A (BPA), has been associated with a number of negative health outcomes. *In vivo*, BPA is rapidly converted to its predominant metabolite, BPA-Glucuronide (BPA-G), which has long been believed to be biologically inactive because it lacks estrogenic activity. However, the effects of BPA-G on cellular metabolism have not been characterized. The current study examines the effect of BPA-G on adipogenesis.

Methods: The effect of BPA-G on the differentiation of human and 3T3L1 murine preadipocytes was evaluated *in vitro* by quantifying lipid accumulation and the expression of adipogenic markers.

Results: Treatment of 3T3L1 preadipocytes with 10 μ M BPA-G induced a significant increase in lipid accumulation, mRNA expression of the adipogenic markers sterol regulatory element binding factor 1 (*SREBF1*) and lipoprotein lipase (*LPL*) as well as the protein levels of LPL, α 2 and adipon. Treatment of primary human preadipocytes with BPA-G also induced adipogenesis as determined by α 2 levels. Co-treatment of cells with the estrogen receptor (ER) antagonist Fulvestrant (ICI) significantly inhibited the BPA-G-induced increase in LPL and α 2 levels, while treatment with ICI alone had no effect. Moreover, BPA-G did not display any significant estrogenic activity.

Conclusions: To our knowledge, this study is the first to show that BPA-G induces adipocyte differentiation and is not simply an inactive metabolite. The fact that BPA-G induces adipogenesis and is inhibited by an ER antagonist yet has no estrogenic activity suggests that it

has no classical ER transcriptional activation function and acts through a pathway that remains to be determined.

Introduction

Environmental chemicals with widespread human exposure such as bisphenol A (BPA) may play an important role in de-regulating normal metabolism (Vom Saal et al. 2012). BPA is used in the manufacture of polycarbonate plastic (Fleisch et al. 2010; Rubin 2011), and has been detected in food, water and dust (Vandenberg et al. 2007). Human exposure to BPA is demonstrated by its ubiquitous detection in blood, urine and adipose tissue (Dekant and Volkel 2008; Vandenberg et al. 2010) and it has been linked to modulation of adipocyte differentiation and obesity (Bhandari et al. 2013; Harley et al. 2013; Masuno et al. 2005; Somm et al. 2009; Wang et al. 2012).

In vivo, BPA is rapidly conjugated to glucuronide giving rise to BPA-glucuronide (BPA-G) after passing through the liver (Knaak and Sullivan 1966; Pottenger et al. 2000). The metabolism of BPA in rat, monkey and human hepatocytes displays some species-specificity; however, BPA-G has been shown to be its major metabolite (Kurebayashi et al. 2010). BPA-G is excreted primarily in the urine in humans and the bile in rodents (Inoue et al. 2001; Inoue et al. 2003; Volkel et al. 2002). Recently, BPA-G has been detected in human serum and urine at higher concentrations than free BPA itself (Harthe et al. 2012; Liao and Kannan 2012; Smith and Saggerson 1979). In addition, BPA-G but not free BPA was detected in blood of humans exposed to low doses of BPA (Volkel et al. 2002). BPA-G has also been investigated in a number of animal models. In rats orally administered with BPA (100 mg/kg) over 6 days, the major form of BPA in the urine, milk and plasma was BPA-G (Snyder et al. 2000). Moreover, pregnant sheep exposed to BPA showed a 1300-fold higher BPA-G accumulation in amniotic fluid and cord blood compared to BPA (Viguie et al. 2013).

It has been the long-held belief that BPA-G, the predominant metabolite of BPA exposure, has no bioactivity and is quickly eliminated *in vivo*. This is mainly due to the fact that BPA is well-known for its estrogenic activity, whereas BPA-G has been shown to lack the ability to activate the ER (Matthews et al. 2001). In HepG2 human hepatoma cells ER α and β activity was not significantly affected following exposure to BPA-G (Snyder et al. 2000). However, free BPA has been shown to possess bioactivity beyond estrogenicity, and therefore it would be reasonable to assume that BPA-G could potentially influence alternate pathways much like BPA. We and others have previously shown that BPA is a significant inducer of adipogenesis in both rodent and human cell models, independent of its estrogenic activity (Boucher et al. 2014; Sargis et al. 2010). BPA has been linked to glucocorticoid receptor (GR) activation as well as the thyroid receptor (TR), androgen-receptor (AR) and estrogen-receptor-related (ERR) γ (Lee et al. 2003; Matsushima et al. 2007; Moriyama et al. 2002; Sargis et al. 2010). The effect of BPA-G on anything other than estrogenic activity and on other receptors has not been determined.

In the current study, the effect of BPA-G on the differentiation of the murine 3T3L1 preadipocyte cell line and primary human preadipocytes was examined. To our knowledge, we show for the first time that BPA-G is not an inactive metabolite and can induce lipid accumulation in both human and murine preadipocytes and increase expression of several key adipogenic markers at the mRNA and protein levels. Moreover, BPA-G-induced differentiation was inhibited in the presence of the specific ER antagonist Fulvesrant (ICI), despite the fact that BPA-G has no estrogenic activity in these cells, suggesting that a potential mechanism of BPA-G action may be through a non-classical ER action or as yet unknown pathway.

Materials and Methods

Adipocyte differentiation. 3T3L1 mouse embryo fibroblasts (ATCC) were cultured according to the supplier's instructions and not used past passage 10. Cells were cultured in DMEM/low glucose media containing 10% bovine calf serum (HyClone Laboratories). Two days after reaching confluence cells were cultured in differentiation medium consisting of DMEM, 10% fetal bovine calf serum (FBS), 500 μ M of 3-isobutyl-1-methylxanthine (IBMX) (Sigma-Aldrich), 100 nM of insulin (Roche Diagnostics), and varying concentrations of BPA-G (Toronto Research Chemicals Inc.) or 250 nM dexamethasone (DEX) (Sigma-Aldrich). Media containing insulin with or without BPA-G were replenished every 2 days. For the ER and GR antagonist studies, 0.01-10 μ M ICI-182780 (ICI) (also known as Fulvestrant) or 1 μ M of the GR antagonist RU486 or 1 nM estradiol (E2) (all Sigma-Aldrich) were also added to the above with or without BPA-G as indicated in each figure. Primary human preadipocytes (Zenbio, Inc.) from donors with body mass indexes \leq 24.99 and who gave informed consent were maintained in Preadipocyte Medium (ZenBio). For differentiation, confluent preadipocytes were treated with media containing 33 μ M biotin, 17 μ M pantothenate (Sigma-Aldrich) and 100 nM insulin for 14 days. In addition, 500 μ M IBMX was also included in the differentiation media from day 0 to day 4. From day 2 until day 14, 5 μ M troglitazone (Sigma-Aldrich) and the indicated concentrations of BPA-G or 1 μ M DEX, as a positive control, were also included in the differentiation media and replenished every 2 days. Ethics approval for the use of primary human adipocytes was obtained by the Health Canada Research Ethics Board.

Lipid staining and quantification. Clear-bottomed black 96 well plates were coated with rat collagen I (Invitrogen) at 5 μ g/ml² in 0.02 M acetic acid for 1 hour at room temperature. 3T3L1

cells were seeded and differentiated as described above for 8 days with the indicated treatments. Cells were then fixed with 10% formalin and stained with Nile Red and DAPI (both 1 $\mu\text{g}/\text{mL}$) in 0.2% Triton-PBS for 15 minutes as previously described (Greenspan et al. 1985). Nile Red (staining for lipid droplets) was viewed at 530 nm and DAPI (staining for cell nuclei) at 405 nm using fluorescence imaging on an Olympus IX71 microscope. Cells were imaged at 100X magnification. Nile Red fluorescence was quantified at 485/528 nm (excitation/emission) and normalized to DAPI staining measured at 360/460 nm using a Synergy 2 Microplate Reader (BioTek Instruments Inc.).

Real-time polymerase chain reaction (PCR). Total RNA was extracted from differentiating cells treated as described using the RNeasy Kit (Qiagen). Genomic DNA was eliminated using the RNase-Free DNase Kit (Qiagen). RNA (250-500 ng) were reverse transcribed into cDNA using iScript Advanced cDNA Synthesis Kit (BioRad). For each real-time PCR reaction, cDNA was amplified in a CFX96-PCR Detection System using the iQSYBR SsoFast EvaGreen Supermix (BioRad). The primer pairs for each gene target were: CCAAT/enhancer-binding protein (*C/EBP*) α : Forward-TAACTCCCCCATGGAGTCGG, Reverse-TATAGACGTCTCGTGCTCGC; *LPL*: Forward-GATCCGAGTGAAAGCCGGAG, Reverse-TTGTTTGTCCAGTGTCAGCCA; *SREBF1*: Forward-CTTTTCCTTAAGGTGGGCCT, Reverse-AGCTGGAGCATGTCTTCGAT; peroxisome proliferator-activated receptor (*PPAR*) γ 1/2: Forward-GCCTGCGGAAGCCCTTGGT, Reverse-GCAGTTCCAGGGCCTGCAGC; *aP2*: Forward-GGAAGCTTGTCTCCAGTGAA, Reverse-GCGGTGATTTTCATCGAATTC; *Adipsin*: Forward-CCTGAACCCTACAAGCGATG, Reverse-CAACGAGGCATTCTGGGATAG; *Perilipin*: Forward-TTGGGGATGGCCAAAGAGAC,

Reverse- CTCACAAGGCTTGGTTTGGC and β -actin: Forward-
GACTTCGAGCAAGAGATGGC, Reverse- CCAGACAGCACTGTGTTGGC. Standard curves
were generated from the pooled cDNA obtained from cells treated with DEX from various time
points. Primer efficiencies were $\geq 90\%$ and specificity was confirmed by sequence blast and
melting curve analysis. Reactions were normalized to β -actin expression which was not affected
by BPA-G treatment.

Western blot analysis. Cells were lysed in RIPA buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1
mM EDTA, 1% sodium deoxycholate, 2% NP-40, 0.4% SDS, 10% glycine) containing protease
inhibitors (Roche Diagnostics). Primary antibodies for aP2 and LPL (R&D Systems), adipsin
(Santa Cruz Biotechnology) and β -actin (13E5) (Cell Signaling Technology) and appropriate
horseradish peroxidase-labelled secondary antibodies were used. Blots were developed using
Clarity Western ECL Substrate (BioRad). Western blots were visualized using ChemiDoc
Imager and quantified using Image Lab software (BioRad). Protein levels were normalized using
 β -actin.

ER and GR transcription assays. 3T3L1 cells plated in triplicate in 12-well cell culture plates
in 10% charcoal stripped serum (Wisent) and phenol red free DMEM (Wisent) media. After 24
hours, the cells were transfected with 3X ERE (Estrogen-responsive element) -TATA-Luciferase
reporter plasmid (ERE-Luc) and ER α and ER β expression plasmids (pVP16-ER α and pVP16-
ER β) (all from Addgene) using Fugene HD (Roche Diagnostics) according to the manufacturer's
instructions. The cells were transfected with 125 ng of the reporter plasmid, 25 ng ER-expression
plasmid and 10 ng pRL-CMV (Promega) used as an internal control. For the GR transcription
assays, experiments were performed in Cos-7 cells which were transfected as above with either

125ng of 3X-GRE-luc (GR-responsive element), or 125 ng of aP2-luc (plasmid containing the aP2 promoter region upstream of luciferase, (Atlas et al. 2014), 25 ng pTL2-GR (a GR expression plasmid), and 10 ng of pRL-CMV as an internal control. Twenty-four hours following transfection, cells were treated with vehicle control, 10 μ M BPA-G, 250 nM DEX, 1 μ M RU486 (GR antagonist), or co-treatment conditions for 24 hours. Cells were then lysed in Passive Lysis Buffer (Promega) and luciferase activity was quantified with the Dual Luciferase Assay kit (Promega) using a Glomax96 Luminometer (Promega). Luciferase activity was normalized to the internal control.

Statistical analyses. Data were analysed by Student's t-test or ANOVA with Holm-Sidak post-test analysis using SigmaPlot 11.0.

Results

Effects of BPA-G on lipid accumulation and expression of adipogenic markers in 3T3L1 preadipocytes. We evaluated the effect of BPA-G on adipocyte differentiation, by assessing lipid accumulation using Nile Red staining and quantification. 3T3L1 cells treated with 0.01-10 μ M BPA-G for 8 days showed increased Nile Red lipid staining, indicating more differentiation compared to control cells treated with vehicle alone (ethanol) (Figure 1A). Cells treated with DEX as a positive control showed roughly 90-100% of the cells positive for lipid staining indicating a high degree of adipocyte differentiation. Quantification of Nile Red staining showed significant lipid accumulation at 10 μ M BPA-G (Figure 1B) inducing a 3-fold increase in fluorescence levels. Cells treated with DEX exhibited an almost 8.3-fold increase in lipid accumulation. No cell death was observed under any of the treatment conditions. To further evaluate BPA-G induced effects on differentiation, mRNA expression of key adipogenic factors

on day 6 post-treatment was evaluated by quantitative real-time PCR in response to increasing doses of BPA-G (0.25-10 μ M). Treatment of cells with 10 μ M BPA-G resulted in a statistically significant increase in the mRNA levels of the adipogenic markers *SREBF1* and *LPL* by 1.5-fold (Figure 1C). The mRNA expression of several adipogenic markers throughout the course of differentiation at days 2, 4, 6 and 8 for all doses of BPA-G as well as for 250 nM DEX (as a positive control) were also measured and modest increases in mRNA levels were observed in *LPL* and *SREBF1* by day 6 following BPA-G treatment (Supplemental Material Figure S1). The data show that BPA-G can induce adipocyte differentiation and lipid accumulation in a time-dependent manner.

Effects of BPA-G on protein expression of adipogenic markers. The effects of BPA-G treatment on the protein levels of the adipogenic markers LPL, aP2 and adipsin were evaluated. The data show that protein levels of the adipogenic markers LPL, aP2 and adipsin were significantly increased on day 8 only at 10 μ M BPA-G and by DEX relative to control (Figures 2A-D). The effect of BPA-G on the differentiation of primary human preadipocytes was also evaluated. The data showed that treatment of cells with 0.05 and 0.25 μ M significantly stimulated adipocyte differentiation as determined by aP2 protein levels (Figures 2E/F). These results clearly show BPA-G induces expression of some key adipogenic markers at both the mRNA and protein levels during adipocyte differentiation in 3T3L1 murine and primary human preadipocytes.

The effects of the ER antagonist ICI on BPA-G-induced differentiation. Given that the parent compound (BPA) has ER binding activity at concentrations as low as 0.1 μ M (Li et al. 2012), the potential role of the ER in BPA-G-induced differentiation was investigated using the

specific ER antagonist ICI. 3T3L1 cells were treated with either vehicle, 1 μ M ICI or 10 μ M BPA-G with increasing concentrations of the ER-antagonist ICI and lipid accumulation was determined. The data showed that ICI alone did not induce significant lipid accumulation whereas 10 μ M BPA-G induced about a 2.5-fold increase (Figures 3A/B). Co-treatment of BPA-G with increasing concentrations of ICI significantly inhibited BPA-G-induced lipid accumulation at 1 and 10 μ M ICI. Protein levels of adipogenic markers were also examined following treatment with 1 nM estradiol (E2) or BPA-G with or without ICI. Co-treatment with ICI significantly inhibited BPA-G-induced LPL and aP2 protein levels by 75% (to background levels) (Figures 3C/D). ICI also appeared to inhibit adipisin expression; however, the decrease in protein levels was not statistically significant due to greater variation in the Western blots. ICI or E2 alone had no effect on differentiation consistent with previous reports (Boucher et al. 2014). We next confirmed that BPA-G does not have estrogenic activity using cells transiently transfected with an ERE-luciferase reporter plasmid. The data show that cells treated with estradiol (E2) as a positive control significantly stimulate ERE-luciferase activity at concentrations of 0.0001-0.01 μ M, whereas BPA-G had no effect on ERE-luciferase activity (Figure 4A). The ability of ICI to inhibit the E2-dependent luciferase activity was confirmed (Figure 4B).

The effects of BPA treatment on GR transcriptional activity. Cos-7 cells transfected with GR and a GRE-luciferase reporter plasmid were treated with either BPA-G or DEX and luciferase activity was quantified. The data show that the GR agonist DEX was able to significantly induce luciferase expression; however, treatment of cells with 10 μ M BPA-G had no effect on GRE-controlled luciferase activity (Figure 4C). An aP2-promoter reporter assay was also used to

evaluate the effect of BPA-G on the ability of GR to regulate the α P2 promoter. The data show that the GR-agonist DEX significantly stimulated α P2 luciferase activity in the presence of GR and that this effect was inhibited by co-treatment with the GR-antagonist RU486 (Figure 4C). However, treatment of cells with BPA-G in the presence of GR did not increase α P2 luciferase activity. Co-treatment of BPA-G with RU486 and RU486 alone had no effect on GR-mediated α P2 luciferase activity. The effect of RU486 on BPA-G mediated adipogenic marker expression was also evaluated by Western blot (Supplemental Material, Figure S2). RU486 on its own up-regulated protein levels of LPL, α P2 and adipsin, as does BPA-G; however, co-treatment of BPA-G and RU486 had no inhibitory effect. Therefore, it is unlikely that BPA-G induced adipogenesis is mediated via the GR.

Discussion

To our knowledge, the current study is the first to show that BPA-G is not an inactive metabolite as previously believed but is in fact biologically active and can induce lipid accumulation and differentiation of preadipocytes in murine and primary human cell models. It has been the long-held belief that the predominant metabolite of BPA exposure, BPA-G is inactive and quickly eliminated *in vivo*. However, the only studies that specifically examined the effects of BPA-G on any cellular or physiological response showed that it had no estrogenic activity, unlike its free precursor BPA which is well known to have weak estrogenic properties (Matthews et al. 2001). However, BPA itself has been shown to be much more than just an estrogenic compound able to influence several signaling pathways such as GR, ERRs, androgen receptor and thyroid hormone receptor (TR), in addition to the traditional ERs (Lee et al. 2003; Matsushima et al. 2007; Moriyama et al. 2002; Sargis et al. 2010). Also, we and others have previously shown that BPA

can induce differentiation of preadipocytes in rodent and human animal models (Boucher et al. 2014; Sargis et al. 2010; Wang et al. 2012). Only one study that the authors are aware of suggested BPA-G may have a physiological effect (Weinberger et al. 2014); however, that study reported the effect of total BPA (both free and BPA-G) and did not distinguish between the two forms. Weinberger *et al.* showed that total BPA (free + BPA-G) concentrations in pregnant mothers were associated with reductions in gestation likely due to BPA/BPA-G-induced alterations in signaling via PPAR γ or androgen precursors during pregnancy, suggesting an effect on the key adipogenic factor PPAR γ (Weinberger et al. 2014). Therefore, it is possible that BPA-G may also bind and exert effects through these other receptors. The fact that we show that BPA-G is an active compound in adipogenesis suggests that it may have effects on other cellular processes warranting further study since BPA-G is the predominant metabolite following BPA exposure.

In this study, we show that the BPA-G induced adipocyte differentiation and mRNA expression of the key adipogenic factors *SREBF1* and *LPL* as well as aP2, LPL and adipsin protein levels. We also show that BPA-G can increase protein levels of the adipocyte marker aP2 in primary human preadipocytes. Future work will further characterize the effect of BPA-G on adipogenesis in these human cells. Consistent with previous reports we also observed no ER-mediated transcriptional activity of BPA-G. Interestingly, the increase in mRNA expression of the key adipocyte transcription factors *CEBP α* and *PPAR γ* in response to BPA-G was not statistically significant, unlike the increase in *SREBF1* and *LPL* expression which play important roles in the determination of the mature adipocyte phenotype. The concentrations of BPA-G used in this study are within the range of those used in other *in vitro* studies and found in human and animal

fluids (Kosarac et al. 2012). BPA-G has been measured in urine of newborn infants in a small study of 12 infants showing the average concentration to be ~ 2 nM (0.87 ng/mL) (Nachman et al. 2013) and was also detected (along with free-BPA and BPA-sulfate) in mid-gestation fetuses in a US population of pregnant women (Gerona et al. 2013). Harthé *et al.* also reported BPA-G in human urine samples with an average BPA-G concentration of 11.5 μ M (4.64 μ g/L) (Harthe et al. 2012), similar to the *in vitro* concentration of BPA-G reported in ER-responsive transcription assays and used in this study (Matthews et al. 2001).

Free BPA has been shown to activate both ER α and ER β (Hiroi et al. 1999) as well as ERR γ (Okada et al. 2008). Previous studies showed that BPA-G did not bind human ER α or ER β *in vitro* nor stimulate ER α or β activity in MCF-7 cells at concentrations of 10 μ M (Matthews et al. 2001). Consistent with these reports, we showed that BPA-G did not stimulate ER-responsive activity in also in 3T3L1 preadipocytes. Surprisingly, BPA-G-induced adipogenesis was inhibited by the ER-antagonist ICI at 1 and 10 μ M concentrations. At 10 μ M ICI, lipid accumulation appears to be inhibited even below background levels (Figure 3A/B); however, at such a high concentration, ICI likely has non-specific effects which may affect the ability of the cells to differentiate. Furthermore, all experiments were completed in 10% FBS which contains some estrogen and estrogen-like compounds. Therefore, ICI would be expected to result in a slight inhibition as compared to control. We have previously reported that ICI was also able to inhibit BPA-induced adipogenesis in primary human preadipocytes (Boucher et al. 2014) despite the fact that estradiol did not have a positive effect on adipocyte differentiation and is considered to be anti-adipogenic (Okazaki et al. 2002). Interestingly, it has been shown that diethylstilbestrol, a potent ER α activator, was adipogenic in mice and 3T3L1 cells and that the

effect could be inhibited by ICI (Hao et al. 2012). The ability of ICI to inhibit the effects of free BPA on ER α and ER β activation was also reported in HepG2 and HeLa cells (Li et al. 2012). The data therefore suggest that BPA-G may assert effects on adipogenesis through an indirect interaction with the ER pathway or an as yet unidentified nuclear receptor that can be inhibited by ICI. One other possibility is that BPA-G may be regulating adipogenesis via nuclear receptors from the estrogen-related receptor (ERR) family which are known to have a role in adipogenesis (Ijichi et al. 2007). ERR α has been shown to up-regulate peroxisome proliferator-activated receptor γ coactivator-1 α (PGC-1 α) expression and PPAR γ /PGC-1 α and SREBF1/PGC-1 α dimers which are important adipogenic transcription factors (Ju et al. 2012). Moreover, free BPA has been shown to bind strongly to estrogen-related receptor γ (ERR γ) (Abad et al. 2008; Matsushima et al. 2007). However, the ability of BPA-G and ICI to affect the activity of ERR proteins has yet to be determined. Some reports have also suggested that free BPA mediates adipogenesis through binding and activation of the GR (Sargis et al. 2010); and we have shown that BPA up-regulated adipogenesis in the absence of glucocorticoids (Boucher et al. 2014). The current study clearly shows that BPA-G can partially replace the effect of DEX, a potent GR agonist, to induce the differentiation of 3T3L1 cells since differentiation is induced in the absence of a GR agonist. Moreover, GR-mediated up-regulation of the α P2 promoter luciferase activity was not increased by BPA-G or inhibited by the GR-antagonist RU486, further suggesting that the effect is not mediated via GR. Free BPA has also been shown to bind TR and the androgen receptors (Lee et al. 2003; Matsushima et al. 2007; Moriyama et al. 2002) which may play roles in adipogenesis and could be potential binding partners for BPA-G warranting further study. Only one study has examined binding of BPA-G to other cellular proteins and that

report evaluated the species-specific differences in transport and metabolism of BPA and BPA-G by rat and human ATP-binding cassette (ABC) transporters which can interact with both chemicals (Mazur et al. 2012). Due to the importance of certain ABC transporters in cholesterol and phospholipid uptake and excretion, perhaps the binding of the transporter to BPA-G or free BPA may play a role lipid metabolism and adipogenesis; however, this remains to be evaluated.

Conclusions

In this study, we show for the first time to our knowledge that BPA-G is biologically active and promotes adipocyte differentiation and lipid accumulation *in vitro*. We also show that while BPA-G does not have estrogenic-activity, BPA-G-induced adipogenesis is still inhibited by an ER antagonist, suggesting the possibility that it may be acting through a non-classical ER action or as yet unidentified pathway. Future studies will examine the potential roles of other nuclear receptors that might bind BPA-G and potentially activate adipocyte differentiation.

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Figure Legends

Figure 1. Bisphenol A-glucuronide (BPA-G)-treated 3T3L1 preadipocytes and the effect on lipid accumulation and adipogenic gene expression. Murine 3T3L1 cells were treated with ethanol (control), BPA-G (0.01-10 μ M) or 250 nM dexamethasone (DEX) as a positive control and lipid accumulation was visualized using fluorescent Nile Red staining (A) and subsequent quantification using a microplate reader (B) on day 8 of differentiation was performed in triplicate. $*P < 0.05$, $**P < 0.001$ relative to control calculated by one-way ANOVA with Holm-Sidak post-test analysis. Scale bar, 200 μ m. (C) Differentiation and treatment of preadipocytes with increasing concentrations of BPA-G was induced as described. Total RNA was isolated on day 6 post-treatment and used for quantitative real-time PCR analysis of the adipogenic markers normalized to β -actin gene expression. Values are expressed as mean fold-change relative to control \pm SEM for 4 experiments. $*P < 0.05$ relative to control calculated by one-way ANOVA with Holm-Sidak post-test analysis.

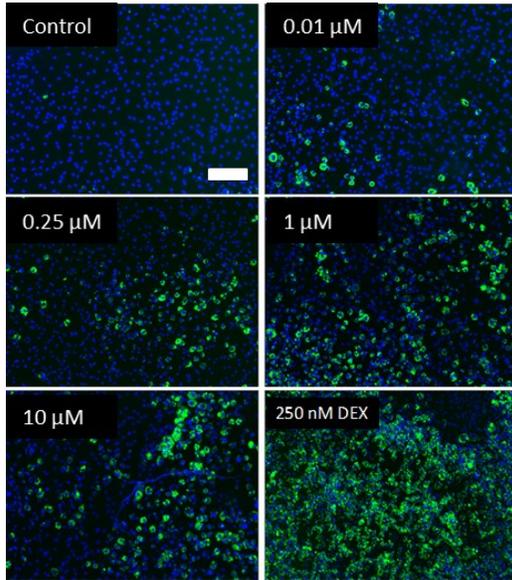
Figure 2. Effect of BPA-G treatment on protein levels of adipogenic markers. Murine 3T3L1 preadipocytes were differentiated as described with the indicated concentrations of BPA-G for 8 days and protein levels of the adipogenic markers LPL, aP2 and adipsin were assessed by Western blot (A) and densitometry (B) analyses. 3T3L1 cells were also differentiated as described with 250 nM DEX as a positive control and protein levels of the adipogenic markers were assessed by Western blot (C) and densitometry (D). Images are representative of at least three separate experiments. β -actin was used as the protein loading control for all blots. All densitometry values are expressed as means \pm SEM of at least 6 experiments. $*P < 0.05$ relative to control calculated by one-way ANOVA with Holm-Sidak post-test analysis. Primary human preadipocytes were treated with ethanol (control) or the indicated concentrations of BPA-G and the protein levels of the adipogenic marker aP2 were assessed by Western blot (E) and densitometry (F) analysis at day 14 of differentiation. β -actin was used as the gel loading control. Values are expressed as means \pm SEM for at least 4 separate experiments. $*P < 0.05$ relative to control calculated by one-way ANOVA with Holm-Sidak post-test analysis.

Figure 3. The effect of the ER antagonist ICI on BPA-G induced differentiation. Preadipocytes were treated with ethanol (control) or 1 nM estradiol (E2) or 10 μ M BPA-G in the presence and absence of increasing concentrations of Fulvestrant (ICI) as indicated and lipid accumulation was visualized (A) and quantified (B) by Nile Red fluorescent staining. Scale bar, 200 μ m. Protein levels of the adipogenic markers LPL, aP2 and adipsin were also assessed by Western blot (C) and densitometry (D) analysis at day 8 of differentiation following treatment with either estradiol (E2), 10 μ M BPA-G, 1 μ M ICI or co-treatment with BPA-G and 1 μ M ICI. β -actin was used as the protein loading control. Values are expressed as means \pm SEM for 6 separate experiments. * $P < 0.05$ relative to control calculated by one-way ANOVA with Holm-Sidak post-test analysis. # $P < 0.05$ relative to BPA-G treatment.

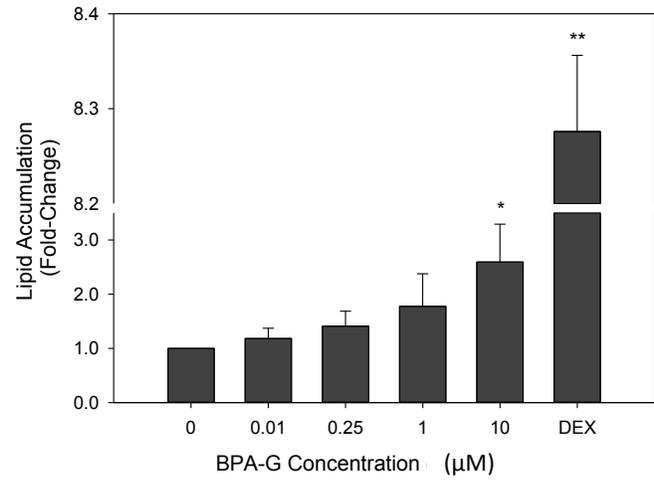
Figure 4. The effect of BPA-G on ER and GR activity. (A) Preadipocytes were transfected with an ERE-luciferase plasmid as well as ER α and ER β expression plasmids for 24 hours then treated with increasing concentrations of estradiol or BPA-G for an additional 24 hours. Luciferase activity was then assessed as described. Values are expressed as means \pm SEM for 3 separate experiments. * $P < 0.005$ relative to untreated control calculated by one-way ANOVA with Holm-Sidak post-test analysis. (B) Preadipocytes were transfected with an ERE-luciferase plasmid and ER α and ER β expression plasmids for 24 hours then treated with 1 nM estradiol or 1 nM estradiol/1 μ M ICI for an additional 24 hours. Luciferase activity was then assessed as described. Values are expressed as means \pm SEM for 3 separate experiments. * $P < 0.05$ relative to untreated control calculated by one-way ANOVA with Holm-Sidak post-test analysis. Cos-7 cells were transfected for 24 hours with a GR expression plasmid (pTL2-GR) and either a (C) 3X GRE-luciferase plasmid or (D) with a reporter plasmid containing the promoter region of aP2 (aP2-luciferase). Cells were then treated with vehicle control (ethanol), DEX, 10 μ M BPA-G with and without the GR antagonist RU486 as indicated. Graphs illustrate fold change over control-treated cells. Values are expressed as means \pm SEM for 3 separate experiments. * $P < 0.05$ relative to control and # $P < 0.05$ relative to DEX-treated cells as calculated by Student's t-test.

Figure 1

A.



B.



C.

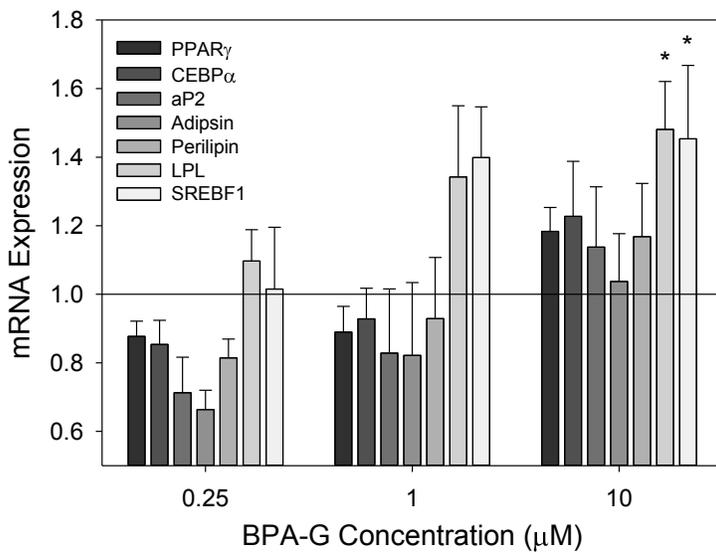
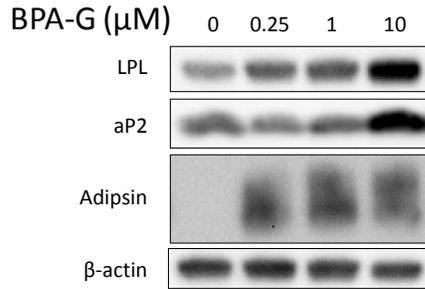
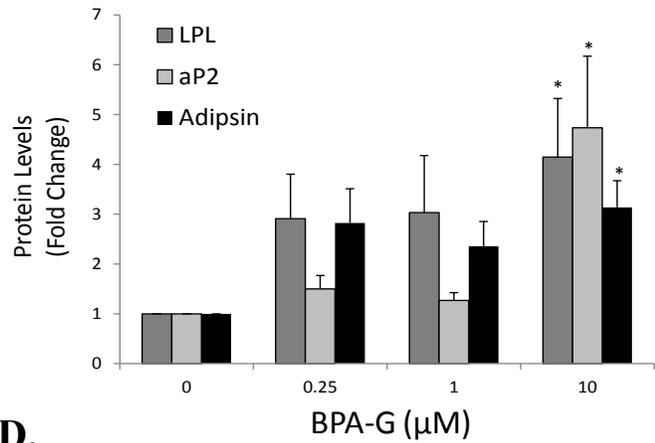


Figure 2

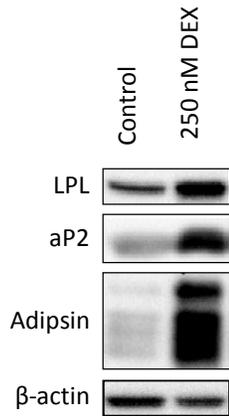
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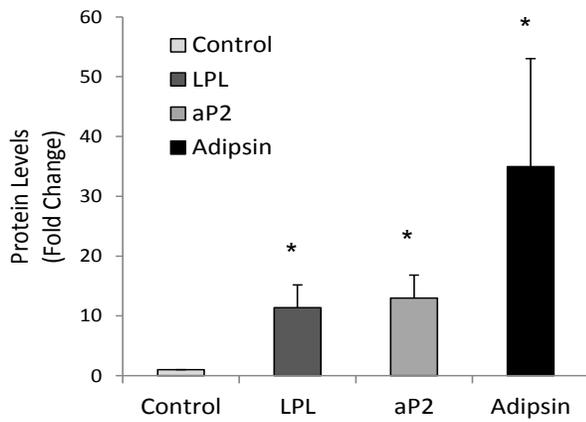
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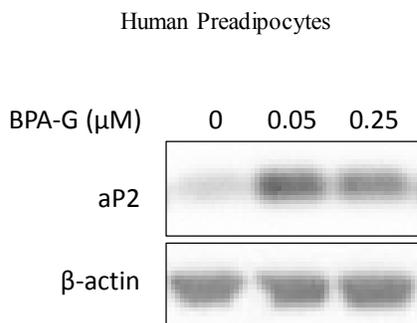
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D.



E.



F.

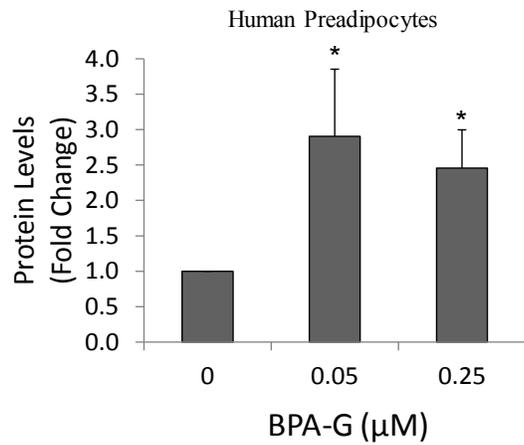
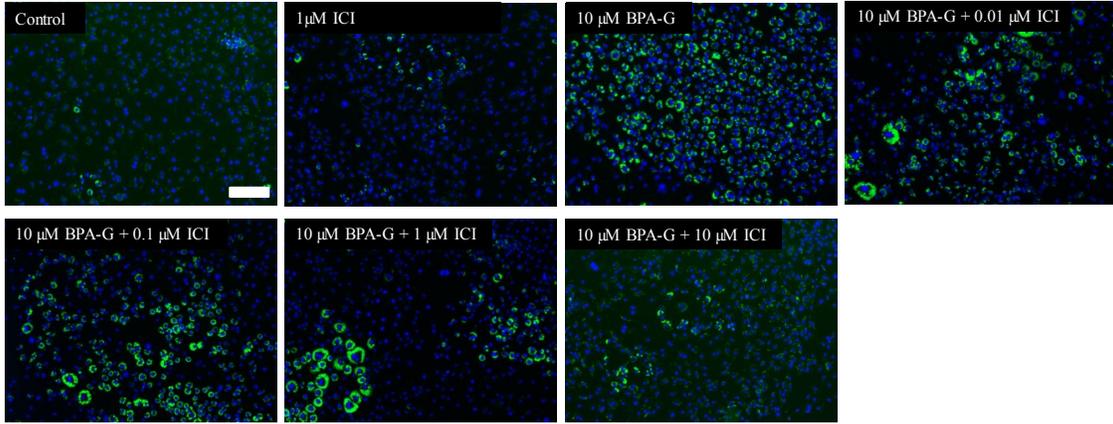
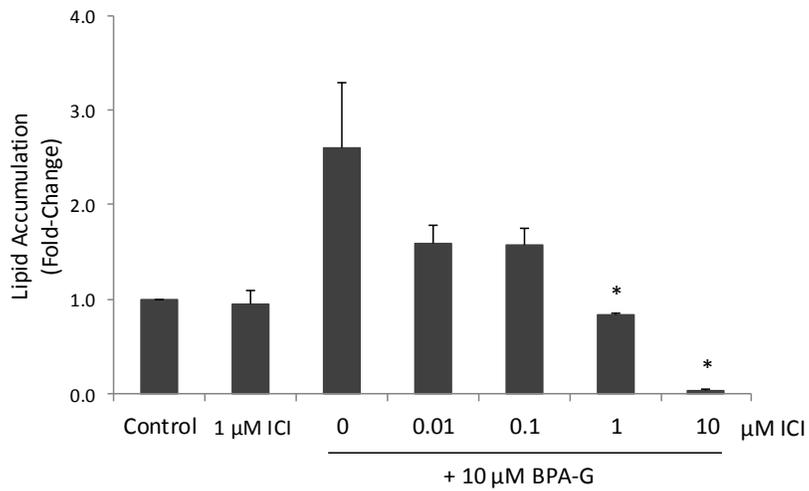


Figure 3

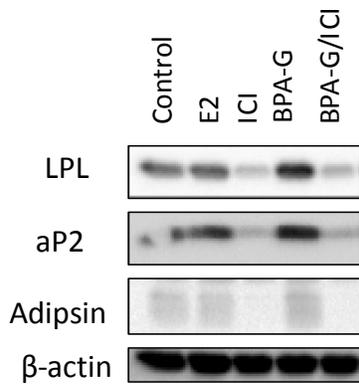
A.



B.



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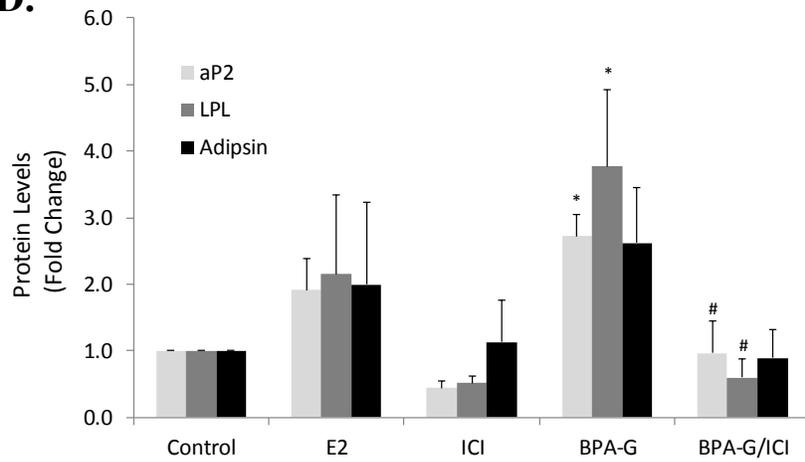


Figure 4

