

Impact of Bisphenol A (BPA) and Free Fatty Acids (FFA) on Th2 Cytokine Secretion from INS-1 Cells

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Received June 17th, 2013; revised July 15th, 2013; accepted August 1st, 2013

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ABSTRACT

Bisphenol A (BPA) is used in huge amounts for many plastic products and is a hormone (estrogen) disrupting agent. BPA as well as FFAs may be deleterious for the immune system. The aim was to identify Th2 cytokines and some of their signal transduction mechanisms in INS-1 cells, an insulin secreting cell line. Screening using a proteome profile indicated an increase of IL-1, IL-2, IL-4, IL-6, IL-10, IL-13 and IL-17 by BPA. Also FFAs (in combination with LPS) were positive. In detailed quantitative measurements, these results were confirmed indicating a complex array of pro- and anti-inflammatory potential. The interaction of BPA with 17 β -estradiol was non-additive with respect to IL-4 and IL-6 release and additive with respect to FFA interaction indicating same and different mechanisms of action, respectively. As signal transduction PI3K (Wortmannin-sensitive) and STAT-3/6 (Tofacitinib-sensitive) are involved in various effects, INS-1 cells release several cytokines due to BPA and FFA attack which may be involved in disturbance of glucose homeostasis and type 1 diabetes.

Keywords: Bisphenol A; Th2 Cytokines; INS-1 Cells

1. Introduction

Bisphenol A (BPA) is the building block of polycarbonate plastic used to make numerous consumer products including baby bottles, beverage containers, food cans (inner layer), Tetra-PakTM, dental materials, thermal paper and impact-resistant safety equipment. BPA is also a part of epoxy resins. It even shows up in cell culture media during cell research when plastic materials are used. 800,000 tons are produced worldwide.

BPA is an endocrine disrupting chemical (EDC) with a toxic influence on reproduction [1]. BPA interacts with G-protein coupled receptors, e.g. GPR-30 and binds to both types of estrogen receptors and various cytokine receptors with low affinity (genomic effect). BPA imitates 17 β -estradiol effects [2] which is not surprising because of the similarity of phenol groups of either compound resulting e.g. in effects on blood glucose homeostasis. BPA disrupts pancreatic beta-cell function [3]. Even low levels can detrimentally affect the glucose metabolism mediated by the glucose-regulated-protein (grp) [4]. BPA is linked to several other diseases such as

allergy, cardiovascular diseases [5] chronic inflammation and e.g. asthma [6]. It modulates immunological processes with respect to differentiation of Th0 cells to Th1 or Th2 cells [7]. BPA increases body weight [8], and since obesity is linked to inflammation [9] (*i.e.* more than 100 hormones and cytokines are secreted by fat cells), this process may be linked to T cells.

BPA is completely (95% - 100%) absorbed [10] and was found in the urine of nearly 100% of investigated children [1,11-13]. Average urine concentration is 3 μ g/L. Plasma concentrations are higher in children than in adults [14] which may partly be due to a slower glucuronidation [10,15,16] making children highly susceptible to damage. In the European Union BPA is not allowed in baby bottles since 2011.

Plasma FFAs play important physiological roles in skeletal muscle, heart, liver and pancreas. FFAs (e.g. palmitate as used in this study) supply energy, build cell membranes and are precursors for intracellular signal molecules such as prostaglandins and leucotriens. Normal concentrations of 200 - 600 μ M are elevated more than three-fold in pathophysiological situations such as obesity and diabetes mellitus [17]. Increased FFAs have

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an immunological potential [18], e.g. inhibiting the T-cell activity. The induction of various proinflammatory effects was described for β -cells [19]. The pancreatic β -cell function and the insulin receptor signaling cascade are inhibited (insulin resistance) [20]. FFA-induced hepatic insulin resistance is associated with increased hepatic diacylglycerol content, increased activities of protein kinase C (PKC), of pro-inflammatory NF κ B pathway and increased inflammatory cytokine expression [21]. FFAs are a major link between obesity and insulin resistance (inhibition of insulin-stimulated glucose uptake), activate PI3K [22] and lead to a chronic inflammatory status [23].

Whereas the influence of BPA on Th1-cytokines is well investigated, not so much is known about Th2, especially not with respect to INS-1 cells, an insulin secreting cell line. The aim of this study is to investigate the impact of pathophysiologically important factors such as BPA and FFA and their complex interplay with 17 β -estradiol with respect to various Th2 cytokines using INS-1 cells. Cytokines which are released by Th2 cells are IL-4, IL-6, IL-10 and IL-13. Also the signal transduction system of cytokines, STAT-3, STAT-6 and Akt, is of interest. The basic question is which pro- and anti-inflammatory cytokines are released from INS-1 cells and which secondary mechanisms are involved. INS-1 cells are a valuable tool since they show physiological and immunological similarities to human pancreas cells [24].

We altogether focus on pathophysiological effects of BPA and FFA on Th2 cytokine secretion from INS-1 cells and some signal transduction mechanisms including interactions with 17 β -estradiol.

2. Materials and Methods

2.1. Chemicals

The proteome profiler was from R&D systems™ (Minneapolis, USA). Photo paper was from Kodak® BioMax™ Light 1, AGFA Rodinal as developer from A&O imaging solutions GmbH, Koblenz, and Tetenal Superfix Plus for fixation from Tetenal AG, Norderstedt, Germany. 24-well plates Cellstar were from Greiner bio-one, Frickenhausen, Germany, and 24-well plates TPP were from Techno Plastic Products AG, Trasadingen, Switzerland. IL assays were from Quantikine® rat ELISA (IL-4, IL-6, IL-10), R&D systems™ (Minneapolis) and RayBiotech™ (Norcross, USA) (rat IL-13, STAT-3 and STAT-6 both phosphorylated and not phosphorylated). Tofacitinib (JAK3 inhibition) was from Pfizer (Berlin, Germany), Wortmannin and palmitate were from Sigma-Aldrich (Hamburg, Germany). All chemicals not mentioned here were of analytical grade and were from PAA, Colbe, or Sigma-Aldrich, Steinheim,

Germany.

2.2. Cell Culture of INS-1 Cells

Asfari *et al.* established in 1992 a X-ray-induced rat transplantable insulin secreting cell line (INS-1). The continuous growth of the INS-1 cells, kindly supplied by Dr. C. Wollheim, Geneva, Switzerland, was found to be dependent on the reducing agent 2-mercaptoethanol to increase the total cellular glutathione levels (Asfari *et al.*, 1992). INS-1 cells with the passage numbers 39-70 were used. They were subcultivated once weekly and the growth medium was changed four days after the subcultivation.

A problem induced by antibiotics added to culture media thus releasing endotoxins from hypothetically present bacteria was circumvented by doing once a while experiments lacking the presence of antibiotics. In fact when the production of endotoxins is decreased by omitting penicillin and streptomycin in culture media, the IL-1 secretion for example was reduced. This has to be kept in mind when the influence of cytokines on initiation of type 1 diabetes is discussed to those experiments.

2.3. Proteome Profiler (ARY 008)

Selected capture antibodies have been spotted in duplicate on nitrocellulose membranes. Cell culture supernatant mixed with a cocktail of biotinylated detection antibodies. The sample/antibody mixture is then incubated. Any cytokine/detection antibody complex present is bound by its cognate immobilized capture antibody on the membrane. Streptavidin-Horseradish Peroxidase and chemiluminescent detection reagents are added, and a signal is produced in proportion to the amount of cytokine bound. The darkening of the photo was scanned and calculated in relative terms (semiquantitative measurement).

2.4. ELISA for IL

The basis is a quantitative sandwich enzyme immunoassay technique (ELISA Quantikine®). A monoclonal antibody specific for various rat interleukins has been precoated onto a microplate. Standards, control (DMSO 1%) and samples are added and any specific cytokine present is bound by the immobilized antibody. After washing away unbound substances, an enzyme-linked polyclonal antibody specific for the interleukin antibody of interest is added to the wells. The enzyme reaction yields a coloured product. Its colour intensity is in proportion to the amount of interleukin bound in the initial step to the first antibody. The sample values were then read off the standard curve which was linear between 31.25 and 2000 pg/mL of either cytokine.

Blanks were between 19.1% and 24.4% of maximum effect.

2.5. Dot Blot of IL-17 Receptor

Washed cells (5 ml cold PBS), lysed in the presence of a protease inhibitor and pelleted (12000 rpm). They were spotted on a nitrocellulose membrane and incubated for 12 hours. After washing the membrane was incubated for 10 min with a primary antibody directed against the IL-17 receptor. Thereafter a secondary antibody linked to horse radish peroxidase, an enzyme which induces the degradation of luminol in the presence of H_2O_2 . The luminescence was detected using a photo paper Kodak[®] BioMax[™].

2.6. Statistics

Results are shown as means + SEM of n independent results. Each of these n results consists of 7 - 9 replicates. Statistical significance was determined using a nonparametric Mann-Whitney test (BBN Software Products Corp.) followed by a post-hoc test (t-test for individual results). $P < 0.05$ was considered significant.

3. Results

In an a priori screening using a Proteome Profiler[®], IL-1, IL-2, IL-4, IL-6, IL-10, IL-13 and IL-17 were highly increased in INS-1 cells by 40 $\mu\text{g/mL}$ BPA (data of 5 $\mu\text{g/mL}$ are not shown, but were rather the same), whereas the effects of FFA [40 $\mu\text{g/mL}$] and LPS [100 ng/mL ; control] were smaller when given alone, albeit higher when both were added in combination (**Table 1**). IL-17 was not increased by FFA and LPS alone (**Table 1**). Many other compounds that are not indicated were positively tested, but only effects on distinct cytokines are shown and only these were evaluated later in more detail.

BPA increased IL-4 secretion in a concentration dependent manner and LPS had the same tendency (**Figures 1(a)** and **(b)**). The effect was time-dependent when data of 6 h (**Figure 1(a)**), 12 h (data not shown) and 24 h incubation (**Figure 1(b)**) were compared. For all further experiments 24 h incubations were preferred.

The IL-4 secretion induced by BPA at three different concentrations was superimposed by 17β -estradiol except at high, possibly maximally effective BPA concentration (**Figure 2(a)**) indicating a right shift of the BPA curve by 17β -estradiol.

The IL-4 secretion induced by FFA at three different concentrations was superimposed by 17β -estradiol (**Figure 2(b)**).

BPA increased the release of IL-6 (**Figure 3(a)**) and 17β -estradiol [100 ng/mL] did not increase this effect at a high BPA concentration (**Figure 3(a)**). 17β -Estradiol

Table 1. Secretion of IL-1, IL-2, IL-4, IL-6, IL-10, IL-13 and IL-17 from INS-1 cells being incubated with LPS, FFA, their combination and BPA. Cells were incubated for 24 h; secretion was determined in a semiquantitative way using a proteome profiler (biotinylated antibodies, nitrocellulose membrane, detection system). Semiquantitative data are indicated: - no secretion, + weak, ++ average, +++ strong secretion. Three replicates with rather identical results.

	LPS 100 ng/mL	FFA 40 $\mu\text{g/mL}$	FFA 40 $\mu\text{g/mL}$ and LPS 100 ng/mL	BPA 40 $\mu\text{g/mL}$
IL-1	+	+	++	+++
IL-1 α	-	-	+	+
IL-1 β	-	-	-	-
IL-1ra	+	-	-	-
IL-2	+	+	++	++
IL-4	+	++	+++	+++
IL-6	-	+	+	++
IL-10	++	+	++	+++
IL-13	+	+++	+	++
IL-17	-	-	+	++

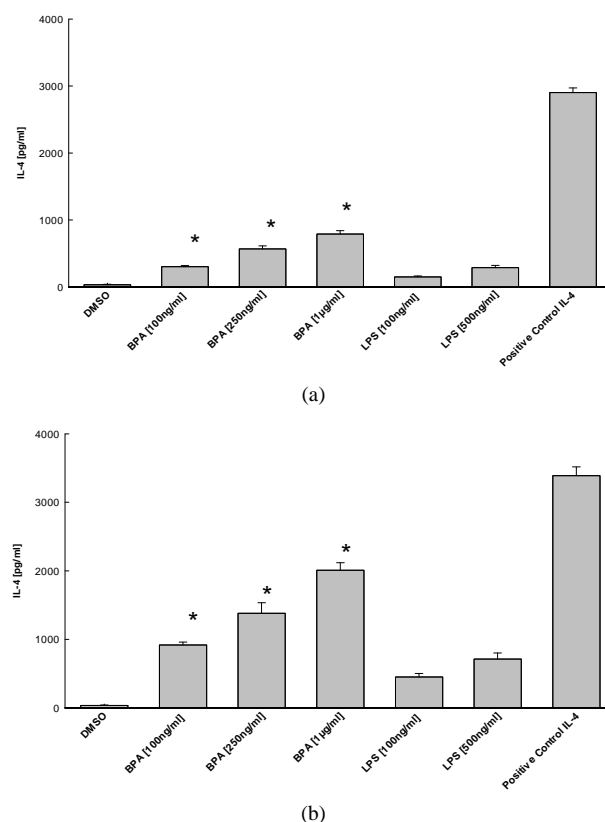


Figure 1. (a) and (b) Effect of BPA and LPS on IL-4 secretion from INS-1 cells. Various concentrations of LPS and BPA were used for a 6 (a) and 24 hour (b) incubation. Blanks (no addition) were subtracted. DMSO (solvent; 1%) and a high concentration of IL-4 [3000 pg/mL] were used as negative and positive controls. Mean + SEM, n = 3; * $p < 0.05$ vs. DMSO control.

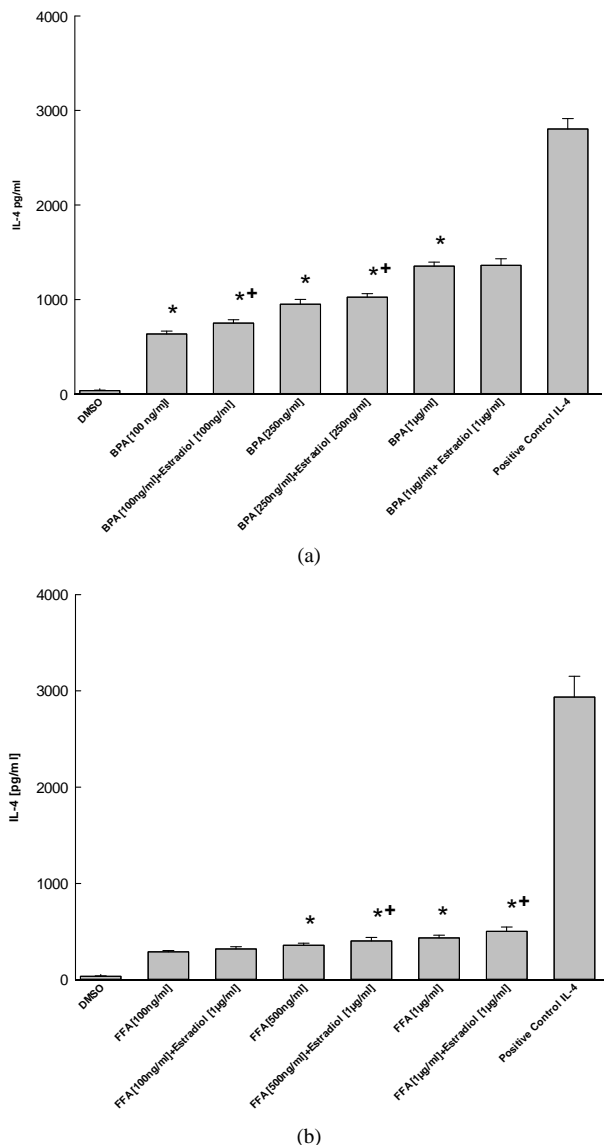


Figure 2. (a) and (b) Modulatory effect of 17β-Estradiol on BPA-induced (a) and FFA-induced (b) IL-4 secretion from INS-1 cells. Blanks (no addition) were subtracted. DMSO (solvent; 1%) and a high concentration of IL-4 [3000 pg/mL] were used as negative and positive controls. Mean + SEM, n = 3; *p < 0.05 vs. DMSO control; **p < 0.05 vs. absence of 17β-estradiol.

[100 ng/mL] increased the release of IL-6 (**Figure 3(b)**) which is superimposed by increasing concentrations of BPA until a maximum BPA concentration of 1.0 μg/mL indicating the same type of mechanism.

Table 2 shows the effect of various concentrations of FFA and LPS on IL-6 secretion. Both compounds increased IL-6 release in a concentration-dependent manner. 17β-Estradiol [100 ng/mL] was not able to increase the effect of FFA at 1 μg/mL.

BPA, LPS and FFA increased IL-10 secretion (**Figure 4(a)**). This effect of BPA at 1 μg/mL was further

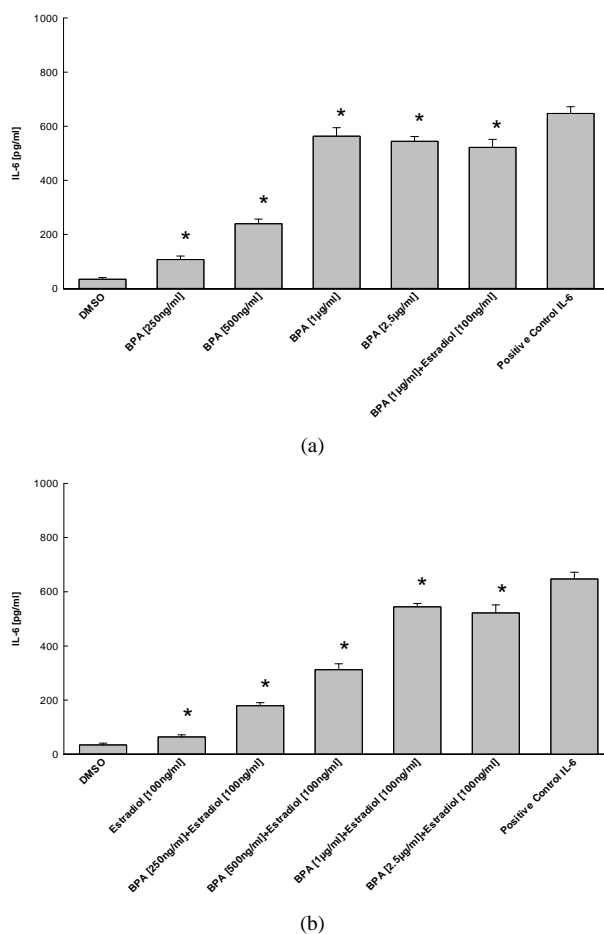
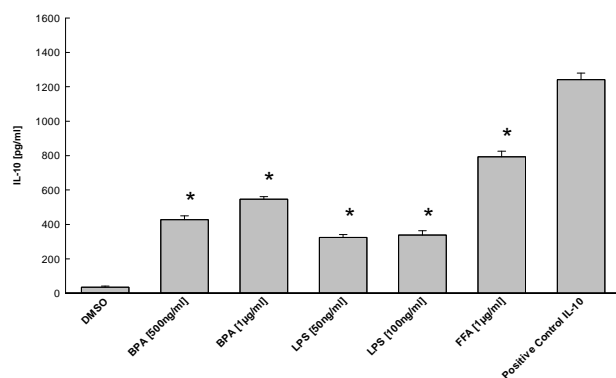


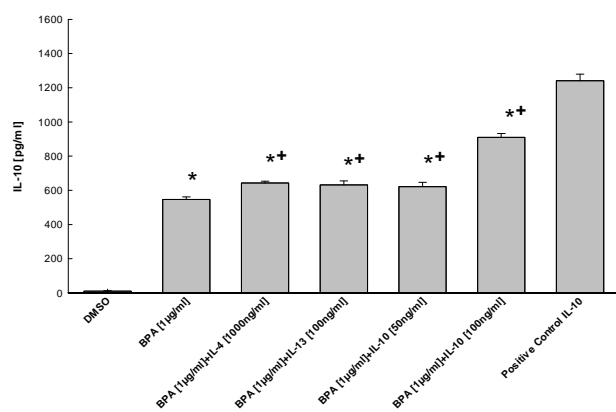
Figure 3. Effect of various concentrations of BPA and a combination with 17β-estradiol on IL-6 secretion from INS-1 cells. 17β-estradiol [100 ng/mL] was combined with various concentrations of BPA [0 to 2.5 μg/mL]. Blanks (no addition) were subtracted. DMSO (solvent; 1%) and a high concentration of IL-6 [650 pg/mL] were used as negative and positive controls. Mean + SEM, n = 4; *p = 0.05 vs. DMSO control.

Table 2. Effect of FFA and LPS on IL-6 release from INS-1 cells (24 h incubation). Details see in the legend of Figure 3.

COMPOUND	IL-6 [PG/ML]
DMSO (0.1%)	14.3 ± 0.4
FFA [0.5 μg/mL]	99.7 ± 8.3*
FFA [1 μg/mL]	197.2 ± 22.4*
FFA [2.5 μg/mL]	276.4 ± 35.6*
FFA [2.5 μg/mL] + 17β-Estradiol [100 ng/mL]	262.6 ± 33.7* (N.S. vs. absence of 17β-estradiol)
FFA [5.0 μg/ml]	296.9 ± 18.7*
LPS [50 ng/mL]	186.3 ± 24.6*
LPS [100 ng/ml]	298.3 ± 16.7*
Positive control: IL-6 [650 pg/mL]	639.2 ± 24.6



(a)



(b)

Figure 4. (a) and (b) Effect of BPA, LPS and FFA on IL-10 secretion from INS-1 cells. Two concentrations of LPS [50 and 100 ng/mL], BPA [500 and 1000 ng/mL] and FFA [1000 ng/mL] were used. Blanks (no addition) were subtracted. DMSO (solvent; 1%) and a high concentration of IL-10 [1250 pg/mL] were used as negative and positive controls. When IL-10 was added, this concentration was subtracted from data shown in column 6 of Figure 4(b). Mean + SEM, $n = 3$; * $p < 0.05$ vs. DMSO control, + < 0.05 vs. BPA effect alone.

increased by IL-4, IL-13 and IL-10 (**Figure 4(B)**) (Note: The added IL-10 has been subtracted from data).

BPA increased IL-13 secretion which was further increased by IL-4 (**Figure 5**).

FFA and LPS increased IL-13 secretion (**Table 3**). The effect of FFA, but not of LPS was inhibited by 1 ng/mL IL-4.

The IL-17 receptor is expressed by INS-1 cells which was verified by a dot-blot experiment shown in **Figure 6**.

BPA and FFA increased p-Akt in a concentration-dependent manner (**Figure 7**). The effect of FFA was inhibited by Wortmannin (PI3K inhibitor) (**Figure 7**).

Figure 8 shows the effect of BPA on both STAT-3 and phosphorylated STAT-3. Both factors are increased by BPA. Tofacitinib (JAK3 inhibition, interferes with JAK-STAT signaling pathway) and wortmannin (PI3K

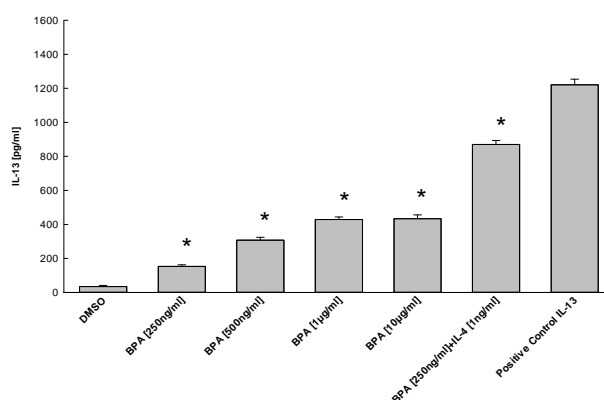


Figure 5. Modulation of the effect of BPA on IL-10 secretion from INS-1 cells; modulators are IL-4, IL-10, IL-13. BPA effect (1 μ g/mL) was modulated by various interleukins, mostly at 100 ng/mL. Blanks (no addition) were subtracted. DMSO (solvent; 1%) and a high concentration of IL-10 (1250 pg/mL) were used as negative and positive controls. When IL-4 was added, this concentration was subtracted from data shown in column 6 of Figure 5. Mean + SEM, $n = 3$; * $p < 0.05$ vs. DMSO control.

Table 3. Release of IL-13 by FFA and LPS and the modulation of this effect by IL-4. Details see in the legend of Figure 5.

Compound	IL-13 [PG/ML]
DMSO (0.1 %)	11.2 \pm 0.3
FFA (250 ng/mL)	76.3 \pm 2.6*
FFA (500 ng/mL)	109.5 \pm 5.3*
FFA [1000 ng/mL]	623.7 \pm 27.1*
FFA [1000 ng/mL] + IL-4 [1 ng/mL] (significant vs. absence of IL-4)	103.4 \pm 8.7*
FFA (10 μ g/mL)	911.6 \pm 19.4*
LPS [10 ng/mL]	203.5 \pm 11.3*
LPS [5.0 ng/mL]	433.9 \pm 9.7*
LPS [100 ng/mL]	834.3 \pm 21.7*
LPS [100 ng/mL] + IL-4 [1 ng/mL]	1087 \pm 41.7*
LPS [1000 ng/mL]	838.9 \pm 19.6*
Positive control: IL-13 [1250 pg/mL]	1241 \pm 28.3

inhibitor) inhibited this BPA effect.

In **Table 4** the effects of various concentrations of BPA, FFA and LPS on STAT-6 expression (phosphorylated and non-phosphorylated) and their inhibition by Tofacitinib (JAK3 inhibitor) and Wortmannin (PI3K inhibitor) is shown.

4. Discussion

4.1. General Comments

The immunological answer to compounds of pathological impact such as the chemical BPA or the

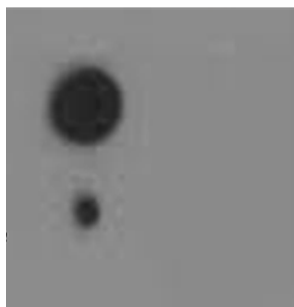


Figure 6. Dot Blot of IL-17 receptor (IL-17R) of INS-1 cells. Details in Methods. Top: sample, bottom: negative control (no addition of primary antibody).

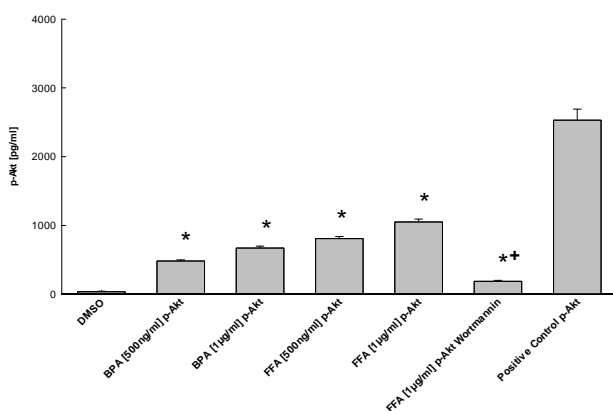


Figure 7. Modulation of Akt (as pAkt) by BPA and FFA and the modulation of FFA effect by Wortmannin in INS-1 cells. Blanks (no addition) were subtracted. DMSO (solvent; 1 %) and a high concentration of pAkt [2500 pg/mL] were used as negative and positive controls. Mean + SEM, $n = 3$; * $p < 0.05$ vs. DMSO control, + $p < 0.05$ vs. absence of Wortmannin.

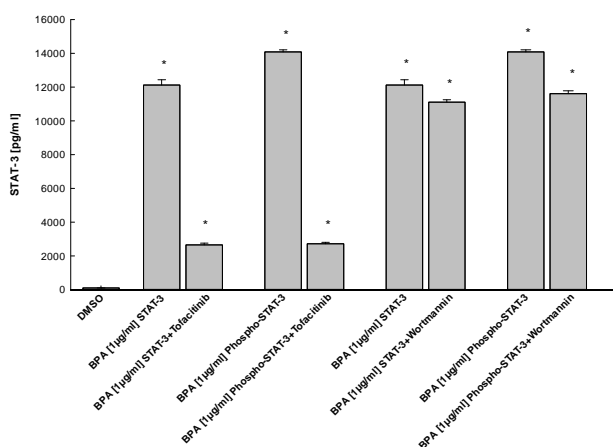


Figure 8. Modulation of BPA-induced activation of STAT-3 (phospho-STAT-3) by Tofacitinib and Wortmannin. INS-1 cells were incubated for 24 hours. Blanks (no addition) were subtracted. DMSO (solvent; 1 %) was used as a negative control. Mean + SEM, $n = 3$; * $p < 0.05$ vs. DMSO control, $p < 0.05$ vs. absence of either inhibitor.

Table 4. Effect of BPA and FFA on STAT-6 and p-STAT-6.

COMPOUND(S)	STAT-6 [PG/ML]
DMSO (0.1 %)	18.4 ± 0.6
BPA (1000 ng/mL)	11030 ± 54.9*
BPA (1000 ng/mL) + Tofacitinib	9311 ± 22.5*+
BPA (1000 ng/mL) + Wortmannin	10760 ± 27.1*
FFA [1000 ng/mL]	8741 ± 51.4*
FFA [1000 ng/mL] + Tofacitinib	7736 ± 39.4*+
FFA [1000 ng/mL] + Wortmannin	8576 ± 35.6*
LPS [1000 ng/mL]	7996 ± 44.6*
LPS [1000 ng/mL] + Tofacitinib	10140 ± 87.6*+
LPS [1000 ng/mL] + Wortmannin	7834 ± 43.6*+
	pSTAT-6 [pg/mL]
BPA (1000 ng/mL)	12360 ± 36.4*
BPA (1000 ng/mL) + Tofacitinib	9624 ± 22.6*+
BPA (1000 ng/mL) + Wortmannin	11120 ± 44.5*+
FFA [1000 ng/mL]	10120 ± 23.6*
FFA [1000 ng/mL] + Tofacitinib	8224 ± 25.4*+
FFA [1000 ng/mL] + Wortmannin	8651 ± 27.9*+
LPS [1000 ng/mL]	9121 ± 33.7*
LPS [1000 ng/mL] + Tofacitinib	10730 ± 76.2*+
LPS [1000 ng/mL] + Wortmannin	8321 ± 44.8*+

metabolic intermediate FFAs is strongly influenced by various cytokines such as IL-4 promoting T helper type 2 (Th2) responses and IL-2 promoting T helper type 1 (Th1) responses. Diabetes and β -cell death originating from various cytokines and transcription factor were well described [25,26]. The Th2 cytokines are not very well investigated with respect to BPA and FFA. Cytokines which are released by Th2 cells are IL-4, IL-6, IL-10 and IL-13. A polarization into the direction of Th2 is partly the answer to processes mediated by IL-4. BPA on a long-term increases insulin levels though not acutely [27]; but the focus here is on proinflammatory cytokines and their balance to the anti-inflammatory cytokines IL-4, IL-10 and IL-13, especially under the influence of BPA and FFA.

17 β -estradiol is of interest because it is known to possess an IL-6 agonistic activity. Interestingly BPA possesses only a low affinity to either estrogen receptor subtype, nevertheless has a potent estrogen-like effect. Estrogen receptors are present on β -cells and promote insulin release [28].

4.2. Screening by a Profiler

When the effects of BPA and FFA were tested using

screening by a Profiler, FFA and BPA induced increases of IL-2, IL-4, IL-6, IL-10 and IL-13 (**Table 1**). The LPS effects as used for control are those as described in the literature except that IL-6 and IL-17 were not secreted. We mainly concentrated on the screened ILs (**Table 1**) except IL-17.

4.3. IL-4

IL-4 is known to influence the differentiation process of Th0 and Th1 which is connected to pathogenesis of type 1 diabetes [29], possibly having a protective effect [30]. BPA increased IL-4 secretion in a concentration- and time dependent manner (**Figure 1**). Thus IL-4, a regulatory cytokine for the adaptive immune response, is regulated by BPA.

17 β -Estradiol had an additional effect to that of BPA (**Figure 2**) which results in a rightward shift of the BPA curve including no effect of 17 β -estradiol at high BPA concentration. This shift indicates an estradiol-like effect of BPA with probably no divergent mechanism of action. There was no superadditive effect between both compounds which would have indicated a different mode of action of both compounds. This is not surprising since BPA is an endocrine disrupting chemical possibly interacting with estrogen receptors as already mentioned. There was a superadditive effect of FFA and 17 β -estradiol.

4.4. IL-6

IL-6 was released by BPA (**Figure 3(a)**) as well as by LPS and FFA (**Table 2**). It is a pro-inflammatory Th2 cytokine being involved in acute phase responses to infection and more important in immune responses. Dysregulation of IL-6-type cytokine signalling is part of inflammation, viral infections and contributes to the onset and maintenance of several diseases, including type 1 diabetes. People suffering from type 1 diabetes show elevated IL-6 levels [31,32]. On the other hand IL-6 protects β -cells from pro-inflammatory effect and functional impairment.

17 β -estradiol is already known to promote IL-6 secretion and to modulate the IL-6 receptor [33]. 17 β -Estradiol increases IL-6 secretion (**Figure 3(b)**); it also increases the effect of BPA, at least at low BPA concentrations (**Figures 3(a)** and **(b)**) indicating the same type of mechanism. This interaction was already obvious for IL-4 (**Figure 2**).

Our data corroborate data on release of IL-6 by FFA from other tissues [34,35]. Interestingly, 17 β -estradiol did not modify the FFA effect on IL-6 (**Table 2**).

4.5. IL-10

IL-10 was secreted by BPA, FFA and LPS (**Figure 4**).

IL-10 is expressed by many cells of the adaptive immune system, including Th2- and T_{reg}-cells and it is well characterized as an anti-inflammatory cytokine with potent suppressive effects in autoimmune diseases [36]. IL-10 function is ambivalent: the absence of IL-10 leads to a better clearance of pathogens with non-enhanced immunopathology, during other infections the absence of IL-10 can be accompanied by a detrimental immunopathology for the host. Permanently increased IL-10 can induce an early type 1 diabetes manifestation [37] but may also produce anti-inflammatory effects in this situation. Elevated IL-10 (e.g. when stimulated by LPS) is correlated with diabetes. IL-10 is known to inhibit the progression of Th1 cells.

Production of IL-10 is regulated in a complex manner: e.g. by the activation of inhibitory pathways and secretion of cytokines from macrophages or by blocking Toll-like receptor mediated MAPK-activation or by IFN- γ inducing the release of glycogen synthase kinase 3 (GSK3) via antagonizing phosphoinositide 3-kinase (PI3K)-Akt activation which leads to inhibition of IL-10 production [38]. IL-10 also negatively regulates p38 phosphorylation and thus limits IL-10 secretion (negative feedback). In INS-1 cells, IL-10 is triggered by IL-4, IL-10 or IL-13 in the presence of BPA (**Figure 4(b)**) which hints at a positive feedback.

4.6. IL-13

BPA, FFA and LPS induce IL-13 secretion from INS-1 cells (**Figure 5, Table 3**). IL-13 is a critical mediator of (allergic) inflammation and shares many functional properties with IL-4 which is obvious by sharing a common receptor unit and common signaling pathways. During the development of type 1 diabetes IL-13 has an inhibitory effect on IFN- γ secretion [39] which is known to be important for diabetes [37]. The situation is complex because IL-13 is a modulator: it inhibits IL-1, IL-6 or IL-12.

4.7. IL-17

IL-17 and its receptor (IL-17R) are basic members of a newly described family of cytokines and receptors, which are different from other cytokine families. IL-17, the hallmark cytokine of the newly defined T helper 17 cell subset, has an ambivalent role by on the one hand protecting the host against extracellular pathogens, and on the other hand in promoting inflammatory pathology in autoimmune diseases such as type 1 diabetes. IL-17 levels are low in diabetes, but are increased by exercise. The impact of IL-17 on the development of type 1 diabetes is discussed and not clear at present [40]. The presence of the IL-17R was demonstrated for INS-1 cells (**Figure 6**). A measurement of IL-17 was not possible at

present, but the profiler experiment has shown a BPA-induced release (Table 1).

4.8. Akt/STAT Signal Transduction

Akt (protein kinase B) is known to modify insulin activity on many enzymes and is activated by PI3K which is involved e.g. in IL-10 action (see above). pAkt stimulated by BPA and FFA may positively act via PI3K which is demonstrated by the inhibitory effect of Wortmannin (Figure 7).

The important cytokines involved in the Th1 and Th2 cell response often trigger Janus kinase (Jak)-STAT signalling pathways, whereas IL-17 family cytokines being different (see above) mediate signalling through the pro-inflammatory NF κ B. Phosphorylated STATs are found in rat pancreatic cells as an answer to released cytokines [41]. STAT-3 is known to be induced by IL-6 and IL-10 (and insulin), STAT-6 by IL-4 and IL-13. Gene knockout studies gave information on STATs being involved in development and function of the immune system. Studies using STAT-6 deficient mice revealed that IL-13 signalling uses the Jak/STAT-6 pathway [42]. Phosphorylated and non-phosphorylated STAT-3 can be activated by a BPA- and FFA-incubation (Figure 8).

Also STAT-6 signal transduction is activated by BPA, FFA and LPS also activate (Table 4). The inhibitory effect of Wortmannin (Figure 7) shows that PI3K may be involved in the BPA effect on Akt. Altogether these signaling pathways are known to be involved in the development of type 1 diabetes.

STAT-6 is an important regulator of inflammation induced by Th2 cells. It is known to be activated by IL-4 and IL-13. STAT-6 is activated e.g. by IL-4 which firstly is phosphorylated as a result. IL-4 also acts on PI3K; STATs and PI3K are used by IL-6; PI3K acts on Akt. Interestingly STAT-3 inhibition by Tofacitinib, a jak/STAT inhibitor, is more pronounced than STAT-6 inhibition (Figure 8 and Table 4).

With respect of INS-1 cells, activation releases a set of primary inflammatory mediators known for acute-phase response such as IL-1, IL-2 and IL-6. We only present here a two-dimensional view which is in reality much more complex: IL-1 for example is known to inhibit IL-6 induced acute-phase protein synthesis in hepatocytes [43]. NF κ B was identified as a mediator for IL-1 dependent suppression of IL-6 in liver cells [44]. The impact of IL-1 towards the secretion of IL-6 in INS-1 cells could not be proven in this work (data not shown).

Data obtained from rodents may be relevant for humans although it has to be admitted that rats are less sensitive to e.g. estrogens. The metabolisms of BPA is similar albeit rats eliminate the glucuronidated products via faeces in contrast to humans (via urine) [45].

4.9. Summary and Conclusion

Altogether BPA, LPS and FFA can increase the release of Th2-cytokines from INS-1 cells (IL-4, IL-6, IL-10 and IL-13) and mediate pro- and anti-inflammatory effects. Data underline the major pathophysiological relevance of BPA and FFA. BPA induces the insulin-stimulated Akt phosphorylation. FFA and BPA activate—probably by one of the investigated released cytokines, STAT-3 and STAT-6 as signal transduction pathways, and also the PI3K/Akt signal transduction pathway. Thus it cannot be excluded that these signals induced by BPA and FFAs may influence development of type 1 diabetes or at least disturb glucose homeostasis. This involvement should be a matter of further detailed investigation.

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