Lifetime-Dependent Effects of Bisphenol A on Asthma Development in an Experimental Mouse Model

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Abstract

Background: Environmental factors are thought to contribute significantly to the increase of asthma prevalence in the last two decades. Bisphenol A (BPA) is a xenoestrogen commonly used in consumer products and the plastic industry. There is evidence and an ongoing discussion that endocrine disruptors like BPA may affect human health and also exert alterations in the immune system. The aim of this study was to investigate age-dependent effects of BPA on the asthma risk using a murine model to explain the controversial results reported till date.

Methods: BALB/c mice were exposed to BPA via the drinking water for different time periods including pregnancy and breastfeeding. To induce an asthma phenotype, mice were sensitized to ovalbumin (OVA), followed by an intrapulmonary allergen challenge.

Results: BPA exposure during pregnancy and breastfeeding had no significant effect on asthma development in the offspring. In contrast, lifelong exposure from birth until the last antigen challenge clearly increased eosinophilic inflammation in the lung, airway hyperreactivity and antigen-specific serum IgE levels in OVA-sensitized adult mice compared to mice without BPA exposure. Surprisingly, BPA intake during the sensitization period significantly reduced the development of allergic asthma. This effect was reversed in the presence of a glucocorticoid receptor antagonist.

Conclusions: Our results demonstrate that the impact of BPA on asthma risk is strongly age-dependent and ranges from asthma-promoting to asthma-reducing effects. This could explain the diversity of results from previous studies regarding the observed health impact of BPA.

Introduction

Asthma is a T helper 2 (Th2) cell-mediated immune response to common environmental allergens and is characterized by airway inflammation with pulmonary eosinophilia, airway hyperreactivity (AHR), and increased serum immunoglobulin E (IgE) levels [1]. Besides genetic predisposition, a strong contribution of environmental factors is reported to be responsible for the increase of allergic diseases in the last decades [2]. First hints from epidemiological studies suggest that amongst others the omnipresent endocrine disruptor bisphenol A (BPA) may be associated with the development of asthma and allergies [3–5]. BPA is commonly used in the plastic industry as well as consumer products like food containers, plastic bottles, thermal paper or dental fillings. Humans are exposed to this compound via ingestion, inhalation and dermal exposure throughout their entire life, including intrauterine life [6–8]. Therefore, the risk of BPA for human health has been discussed intensively in recent years. Previous data from epidemiological as well as animal studies suggest that BPA may affect the reproductive system, insulin production, mental and motor development, and the immune system [9,10]. In particular, maternal exposure with its subsequent effects on disease risk in the offspring is of increasing interest since this period seems to be critical for the priming of the immune system [11]. However, the results from epidemiological studies and experimental animal models regarding the potential risk of BPA exposure on airway inflammation are highly diverse. Although some studies demonstrate asthma/wheezing-promoting effects of BPA [3,4,12,13], inverse associations between urinary BPA levels and wheezing or even a Th1-increasing impact have also been reported [5,14,15]. Only some of the in vitro approaches provide data regarding the possible mechanisms for the immunomodulatory effects of BPA. The studies mainly describe an interference with hormone receptors like the estrogen receptor. However, these data are also inconsistent and show contrasting results [16,17]. The inconsistency in the findings might arise due to different observation periods in human studies and the diverging design of the animal models, including the use of varying BPA doses and the route of exposure [18].

Since a direct causality between BPA exposure and an increased risk for asthma development cannot be studied in humans, we chose an experimental mouse model, which displays all hallmarks
of the human disease, like eosinophilic inflammation of the lung, airway hyperreactivity and increased IgE levels [19]. Although differences in the metabolism of xenobiotics exist between humans and rodents, a similarity of BPA pharmacokinetics in women and mice was shown previously [20]. Therefore, in the present study we have investigated the effects of BPA exposure for different time periods during the whole life span on asthma development in an experimental mouse model. Our findings reported here, demonstrate that only a lifelong BPA exposure starting at birth exacerbated the allergic airway inflammation, whereas maternal

Figure 1. Prenatal BPA exposure did not affect the asthma phenotype in the offspring. Mice were exposed to 5 μg/ml BPA via drinking water during pregnancy. In the offspring an asthma phenotype was induced by sensitization to ovalbumin (OVA) followed by an intrapulmonary allergen challenge as described in Materials and Methods. BPA exposure did neither affect total cell number in BAL fluid (a), lung inflammation (b), lung resistance (c), OVA-specific IgE serum levels (d) nor cytokine production in splenocytes (e) or lymph node cells (f). Data are expressed as mean ± SEM, n=11 animals per group. doi:10.1371/journal.pone.0100468.g001
exposure showed no effect on the disease outcome in the offspring. In fact, a BPA exposure of the adult mice even led to a reduced allergic immune response. These results imply that effects of BPA on allergic immune responses clearly depend on the time point of exposure during development and may range from asthma-suppressing to asthma-promoting impacts.

Methods

Mice

Female BALB/cByJ mice (6–8 weeks of age) were obtained from the Elevage Janvier Laboratory (Le Genest St Isle, France). Mice were bred and maintained in the animal facility at the University of Leipzig (Germany) under conventional conditions with 23°C room temperature, 60% humidity, and 12 h day/night rhythm. Control and BPA-exposed dams and pups were housed in polyphenylsulfone (PPS) cages (PPS can withstand very high

Figure 2. Perinatal BPA exposure showed no effect on the asthma phenotype in the offspring. Mice were exposed to 5 μg/ml BPA via drinking water during pregnancy and breastfeeding. In the offspring an asthma phenotype was induced by sensitization to OVA followed by an intrapulmonary allergen challenge as described in Materials and Methods. BPA exposure did neither affect total cell number in BAL fluid (a), lung inflammation (b), lung resistance (c), OVA-specific IgE serum levels (d) nor cytokine production in splenocytes (e) or lymph node cells (f). Data are expressed as mean ± SEM, n=22 animals per group. *P<0.05.
temperatures without breaking down or releasing ingredient chemicals [21] and bedded with LIGNOCEL bedding material. All mice received phytoestrogen-free diet (C1000 from Altromin, Lage, Germany) and water ad libitum from custom-built glass bottles to avoid contamination with BPA. Experiments involving an asthma induction included groups of 4–6 mice/cage and were performed at least 2 times according to institutional and state guidelines.

Ethic statements
Animal protocols used in this study were approved by the Committee on Animal Welfare of Saxony/Leipzig ( Permit Number: TVV10/10). Buffy Coats for isolation of peripheral blood mononuclear cells were obtained from healthy human donors (Institute of Transfusion Medicine, University of Leipzig, Germany) with fully informed written consent, conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the University of Leipzig (Permit Number: 155–2008).

Exposure to BPA and OVA Immunization
BALB/c mice were exposed to BPA (5 \( \mu \)g/ml) exclusively via drinking water (custom-built glass bottles) for different time periods. Each exposure protocol was performed at least 2 times. To investigate the impact of an intrauterine exposure on the development of allergic airway inflammation in the offspring we exposed pregnant mice to BPA using our established transgenerational asthma model [22–24]. Briefly, female mice were exposed to BPA via drinking water one week before mating, though exposure was discontinued during the mating period of 1 week. Control dams received normal drinking water. Afterwards, BPA or normal water was given to pregnant mice either until delivery (prenatal exposure) or until weaning when pups were 3 weeks old (perinatal exposure). Just before delivery pregnant mice were separated into single cages. In a different approach mice were exposed to BPA via the same route starting after delivery during breast-feeding and offspring were further exposed during lifetime. Exposure of adult mice to BPA or normal water started one week before the first allergen injection and was continued until the end of the asthma induction protocol. To induce an allergic asthma the 6-week old offspring from at least 3 mothers (2–6 mice/group, at least 2x induction protocol. To induce an allergic asthma the 6-week old offspring from at least 3 mothers (2–6 mice/group, at least 2x

Measurement of airway responsiveness
Lung resistance (R\(_L\)) was measured by invasive plethysmography in response to inhaled methacholine (Sigma-Aldrich) as described previously [23,28]. Therefore, mice were anesthetized (100 mg/kg ketamine and 10 mg/kg xylazine, Bayer, Leverkusen, Germany), intubated, and mechanically ventilated at a tidal volume of 0.2 ml and a frequency of 150 breath/min. Baseline R\(_L\) and responses to aerosolized saline (0.9% NaCl) were measured first, followed by responses to increasing doses (2.5 to 40 mg/ml) of aerosolized methacholine.

Collection of bronchoalveolar lavage (BAL) fluid
All cells within the lavage fluid were counted using a hemocytometer. Diff-Quik (Medion Diagnostics AG, Dudingen, CH) stained cytopsins were differentiated into eosinophils, macrophages, lymphocytes and neutrophils according to morphological criteria as described previously [22,29].

Lung histology and computer-based quantification of inflammation
Left lung was fixed in 10% formalin and stained with Haematoxylin & Eosin (H&E, MERCK, Darmstadt, Germany). For quantification and objective evaluation of the degree of histological inflammation, lung sections were scanned with a digital camera (Zeiss, 5 shots per lung) and analysed with HistoClick-Software based on morphometric image analysis [25,30]. The degree of inflammation is expressed as the percentage of pixels which correlate to the stained cells of interest.

OVA-specific IgE assay
OVA-specific IgE serum levels were measured by sandwich ELISA according to a standard protocol as described previously [25].

Cytokine production
One day after airway function test splenocytes or mediastinal lymph node cells (5x10\(^6\) cells/ml per well) were isolated and re-

| BPA (\( \mu \)g/ml)* | Animals | N | Mean (ng/ml) | Range (ng/ml) |
|-------------------|---------|---|--------------|---------------|
| 0                 | Adult   | 7 | 2.27±1.22    | 0.69–3.77     |
| 0.5               | Adult   | 9 | 8.46±4.7**   | 3.21–15.83    |
| 5                 | Adult   | 13| 19.17±9.12***| 7.64–44.13    |
| 25                | Adult   | 9 | 37.19±18.95***| 19.02–79.51   |
| 0                 | Pup\(^a\) | 7 | 4.31±2.63    | 1.77–8.19     |
| 5                 | Pup\(^a\) | 7 | 23.99±7.26***| 17.18–39.43   |

*BPA drinking water concentration.
**P<0.01, ***P<0.001 compared to non-exposed control.
Mother mice were exposed during pregnancy and breastfeeding and adult mice for 5 weeks to BPA via drinking water. BPA serum levels of adult mice or pups were measured at the end of the exposure period as described in Methods.

Table 1. BPA levels measured in serum.

doi:10.1371/journal.pone.0100468.t001
stimulated in vitro with 200 μg/ml OVA in culture medium (RPMI medium supplemented with 10% FCS, 100 U/ml Penicillin, 100 μg/ml Streptomycin). After three days of culture cytokines were measured in supernatants using DuoSet ELISA kits (R&D Systems, Minneapolis, USA) according to the manufacturer's instructions.
BPA ELISA

BPA concentration in serum was detected with BPA Assay Kit (Immuno-Biological Laboratories, Hamburg, Germany). Serum samples, enzyme-labeled BPA and anti-BPA serum were added to a pre-coated microtiter plate with anti-rabbit IgG and incubated for 1 hour at room temperature. After washing, TMB was added as substrate and colour reaction was detected at 450 nm. BPA serum concentration was calculated from a standard curve with a detection range from 0.3–100 ng/ml.

Figure 4. Exposure of adult mice to BPA during sensitization reduced the allergic immune response. Adult mice were exposed to 5 μg/ml BPA via drinking water during OVA-immunization. BPA exposure reduced total cell number in BAL fluid (a), lung inflammation (b), lung resistance (c), OVA-specific IgE serum levels (e) and Th2 cytokine production from lymph node cells (f), while cytokine production from spleen was not affected (e). Data are expressed as mean ± SEM, n=18 animals per group. *P<0.05.

doi:10.1371/journal.pone.0100468.g004
Culture and treatment of murine bone marrow-derived dendritic cells

Bone marrow cells were isolated from naive mice and grown in RPMI medium containing 10% FCS and 20 ng/ml rmGM-CSF (Sigma) for 7 days. Some of the cells were cultured in the presence of BPA and maturation was induced by adding 1 μg/ml LPS (Sigma) for 24 hours at day 7 of culture. Cells were characterized by staining with an anti-CD11c–Phycoerythrin (PE) mAb. The supernatant was collected for IL-12 cytokine measurement according to manufacturer’s instructions (DuoSet ELISA kits, R&D Systems).

Culture and treatment of human monocyte-derived dendritic cells

CD14+ monocytes were isolated with CD14 microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) from peripheral blood mononuclear cells of healthy donors and cultured in the presence of 100 U/ml IL-4 (PeproTech GmbH, Hamburg, Germany) and 1000 U/ml GM-CSF (Berlex, Richmond, VA, USA) for 5 days. Different BPA concentrations were added during the differentiation. To induce maturation 300 ng/ml LPS were added and IL-12 was measured in supernatants according to manufacturer’s instructions (IL-12 ELISA Ready-SET-Go!, eBioscience, Frankfurt, Germany).

Statistical analysis

Mann-Whitney-U and Wilcoxon signed-rank test were used to determine statistical differences between groups. Data were expressed as mean ± SEM and P values of less than 0.05 were considered significant.

Results

Pre- and Perinatal Exposure to Low-dose BPA did not Affect the Asthma Outcome in the Offspring

OVA-sensitized offspring from three mothers exposed to BPA during gravidity did not develop an exacerbated allergic airway inflammation or an increased AHR compared to sensitized mice from three un-exposed mothers (Fig. 1A–F, offspring n=11). Similar results were observed in offspring from dams exposed to BPA during both, gravidity and lactation (6 exposed or non-exposed mothers, offspring n=22). A slight increase in lung inflammation, OVA-specific IgE serum levels and Th2 cytokine levels in the lymph nodes were observed, only IL-13 secretion was significantly increased (Fig. 2A–F).
Lifelong exposure to BPA significantly increased the allergic airway inflammation

In the next experimental set-up three nursing mothers were exposed to BPA, whereas control mice (3) received only drinking water. After weaning the offspring (n=6) were further exposed until the last allergen challenge. First, we measured BPA serum levels in adult mice as well as in their pups after an exposure for 21 days. BPA concentration reached 19 ng/ml in adult mice exposed to drinking water containing 5 μg/ml BPA. We could also detect BPA in the serum of the pups nursed by exposed mothers with a mean of 23 ng/ml (Table 1).

OVA-sensitized mice exposed to BPA as described above revealed an enhanced number of eosinophils in the BAL fluid compared to non-exposed control mice (Fig. 3A). Lifelong BPA intake also exacerbated airway inflammation in the lung (Fig. 3B) and AHR (Fig. 3C). Furthermore, serum levels of OVA-specific IgE were clearly enhanced (Fig. 3D), whereas we observed only a slight but not significant increase of Th2 cytokine levels in re-stimulated splenocytes and lymph node cells of BPA-exposed OVA-sensitized mice (Fig. 3E-F).

To examine whether BPA may affect the differentiation and function of dendritic cells (DC), which play a crucial role in processing environmental signals, we differentiated mouse and human DC from precursor cells in vitro. The presence of BPA during differentiation reduced the production of IL-12 after LPS-stimulation in both, murine and human DC (Fig. S1).

Exposure of adult mice to BPA during sensitization reduced the allergic immune response

Surprisingly, BPA exposure of adult mice during OVA-sensitization diminished the number of eosinophils in the BAL fluid compared to non-exposed control animals (Fig. 4A). Accordingly, histological examination of H&E stained lung sections revealed a significantly decreased inflammation in the BPA-exposed group compared to control mice (Fig. 4B). BPA exposure also prevented the development of methacholine-induced AHR in BALB/c mice (Fig. 4C) and significantly reduced OVA-specific IgE levels compared to non-exposed OVA-sensitized control mice (Fig. 4D). In contrast, Th2 cytokine levels were only diminished in the lymph nodes but not in splenocytes (Fig. 4E-F).

To investigate whether different BPA exposure levels might induce alternate effects, we treated adult mice with two additional BPA concentrations (0.5 μg/ml or 25 μg/ml). BPA exposure to 25 μg/ml led to an even more reduced asthma phenotype in the OVA-sensitized adult BALB/c mice, whereas exposure to 0.5 μg/ml had no effect (Fig. S2).

Glucocorticoid Receptor Antagonist RU486 Abolished the Decreased Immune Response Induced by BPA

Since it has been shown that BPA can bind to the glucocorticoid receptor (GR) as an agonist [31,32], we tested whether blocking this receptor would abolish the BPA-induced suppressive effect on the allergic immune response. Treatment of mice with the GR antagonist RU486 reversed the decreased allergic immune response induced by BPA leading to an eosinophilic airway inflammation, AHR and OVA-specific IgE levels comparable to OVA-sensitized control mice (Fig. 5A-D).

Discussion

Although in recent years several experimental studies have addressed the question, whether BPA may affect the development and the outcome of allergic diseases, the overall picture did not become clearer. Whereas some studies showed a BPA-induced increase of Th2-driven immune reactions with enhanced IgE and IL-4 cytokine levels in adult mice [33,34], others could demonstrate an augmentation of Th1-mediated responses with rather decreased IgE levels [14,15]. In further studies, maternal BPA exposure came into focus, since it has been suggested that the prenatal period represents a critical window in which the developing immune system may be primed towards a specific immune phenotype [11]. However, the data concerning prenatal BPA exposure are diverse, ranging from increased asthma susceptibility [12,33] to an elevated Th1 immune response [36] and no effects [37]. These striking differences might be mainly caused by the used BPA doses ranging from 0.5–5000 μg/kg/day and diverse exposure routes resulting in different bioavailability and metabolism of BPA [30]. In the present study, we exposed mice for different time periods during the whole lifespan to 3 μg/ml BPA via the drinking water. This was half of the dose used in experimental mouse models demonstrating an asthma-promoting effect in the offspring after maternal BPA exposure [12,33]. Assuming a water intake of the mice between 3 and 5 ml/day [39] the BPA dose/day was about 15–25 μg. This is 10 to 20 times higher than the “No Observed Adverse Effect Level” (NOAEL, 50 μg/kg/day). However, the concentration detected in serum of adult mice in this study was comparable to detected BPA levels of up to 19 ng/ml in maternal serum [40]. Despite using the limited ELISA method in comparison to HPLC-mass spectrometry, we could also find elevated BPA serum levels in pups nursed by exposed mothers, which is in line with human studies showing that BPA may cross the placental barrier and can also be detected in breast milk [6]. However, in the present study we could not observe an effect on the asthma outcome in the offspring after maternal BPA exposure, although a trend was detectable in particular after perinatal exposure. One could assume that levels twice as high (10 μg/ml) would exert a similar asthma-promoting impact in our model as described by others using a BPA concentration of 10 μg/ml [12,33]. On the other hand, we speculate that the daily exposure of 30–50 μg BPA (assuming 3–5 ml water intake/day) will probably result in much higher BPA concentrations than the described maximum level of 20 ng/ml in human serum [6].

Whereas no effect was observed after pre- and perinatal BPA exposure, an increased allergic airway inflammation was found when BPA was given from birth until the end of the asthma protocol. Interestingly, similar results were found in a recent study from Donohue et al. investigating BPA exposure and asthma development among inner-city children [5]. The authors showed a positive association between postnatal urinary BPA concentrations and wheeze at ages of 5 and 6 years, whereas prenatal BPA exposure was inversely associated with wheeze at age of 5 years. Although this is in line with our results, there are contrary findings from Spanier et al. demonstrating an association of prenatal BPA exposure and increased odds of child’s wheeze [5]. Donohue et al. explained the differences with the study design: assessing BPA exposure during the last trimester, whereas Spanier and colleagues measured BPA exposure during the second trimester. The second trimester encompasses formation of the terminal bronchioli and the initiation of antigen-specific immune responses [41] and therefore, could be a critical period for airway and immune development. In contrast, others have identified the third trimester as a critical window of fetal susceptibility for BPA effects on lung development [42]. However, it is quite unlikely that an exposure to BPA will be limited to such an exact period. Furthermore, this
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... explanation fails in respect to our model since we exposed the mice during the whole gravidity.

Either way, only the developing immune system during early life seems to be susceptible to an asthma-promoting impact of BPA because an exposure to this compound in later life led to opposite effects in our model. However, there are only very limited information about underlying mechanisms explaining the Th2-supporting properties of BPA. Previous studies have demonstrated that pollutants may interfere with IL-12 production leading to a decreased Th1 differentiation [28,43]. Using murine as well as human DC differentiation cultures we could also observe diminished IL-12 levels in the presence of BPA. This was similar to findings by Guo et al., who demonstrated that BPA-exposed DC produced higher levels of IL-10 relative to that of IL-12 and preferentially induced Th2 deviation [16]. However, based on this assumption exposure of the adult mice during antigen sensitization should also enhance the allergic immune response. Though, BPA exposure at this time period resulted in a completely different outcome. Here, BPA induced a significant attenuation of eosinophilic airway inflammation, AHR and antigen-specific IgE levels.

There are several mouse studies demonstrating an increased Th1 reactivity [14,15], whereas we could not find any signs for a modified Th1/Th2 balance. To clarify, whether BPA might induce a worsened disease outcome at higher concentrations adult mice were exposed to 25 μg/ml. However, even at this BPA dose the allergic airway inflammation was diminished. Since we found no evidence for a Th1/IFN-γ-driven suppression of the allergic immune response as reported before [44,45], we investigated a possible involvement of the previously described BPA-sensitive glucocorticoid receptor [31,32]. As glucocorticoids belong to the standard therapy of asthmatics and also reduce asthma parameters in murine asthma models [46], we blocked an agonistic interaction of BPA with the glucocorticoid receptor by treatment with the antagonist RU486. The inhibition of this signaling pathway reversed the asthma prevention by BPA. Therefore, an involvement of the glucocorticoid receptor could explain the immune suppressive effects of BPA during antigen sensitization. It should be noted that BPA is also described as an estrogen-receptor agonist [47] and may exert its effects via this pathway. However, information from the experimental studies that have investigated the role of estrogen receptor activation in asthma development is contradictory and showed both Th2 cell stimulating effects and anti-inflammatory properties [47,48]. Therefore, we did not analyze a possible involvement of the estrogen receptor in our study.

To evaluate the actual risk of BPA exposure for human asthma, in particular by conclusions based on the use of animal models, the study.

analyze a possible involvement of the estrogen receptor in our anti-inflammatory properties [47,48]. Therefore, we did not contradictory and showed both Th2 cell stimulating effects and the role of estrogen receptor activation in asthma development is also enhanced. AHR and antigen-specific IgE levels.

Supporting Information

Figure S1 BPA exposure during DC differentiation impaired IL-12 production. BPA was added during differentiation of murine bone marrow-derived dendritic cells (a) and human monocyte-derived dendritic cells (b). IL-12 production was measured 24 hours after maturation with LPS. Data are expressed as mean ± SEM, n=4. *P<0.05, **P<0.001 compared to control. (TIF)

Figure S2 BPA exposure during OVA-sensitization reduced allergic airway inflammation in a dose-dependent manner. Adult mice were exposed to BPA via drinking water during OVA-immunisation protocol. BPA exposure reduced total cell number in BAL fluid (a), OVA-specific IgE serum levels (b) and lung resistance (c). Cytokine production from CD4+ lung T cells was not affected (d). Data are expressed as mean ± SEM, n≥7 animals per group. *P<0.05, **P<0.01 of OVA+5 μg/ml BPA and ***P<0.05 of OVA+25 μg/ml BPA compared to OVA. (TIF)

Acknowledgments

We thank Marita Reiprich and Michaela Loschinski for excellent technical assistance and Animesh Bhattacharya for critical reading of the manuscript.

Author Contributions

Conceived and designed the experiments: SP MA TP. Performed the experiments: SP. Analyzed the data: SP MA TP. Contributed reagents/materials/analysis tools: MA JS IL TP. Wrote the paper: SP MA JS IL TP.

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PLOS ONE | www.plosone.org 9 June 2014 | Volume 9 | Issue 6 | e100468
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