



The plasticizer dibutyl phthalate (DBP) potentiates chemical allergen-induced THP-1 activation

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ABSTRACT

Recent studies suggest that phthalates may have a role in the development of allergic diseases, probably due to an adjuvant effect. The present study aimed to investigate the possible adjuvant effect of dibutyl phthalate (DBP) in two in vitro models of contact-allergen induced cell activation, namely the NCTC 2544 IL-18 assay and the THP-1 activation assay. Results show no adjuvant effect in the human keratinocyte cell line NCTC 2544, indicated by lack of increase in interleukin 18 (IL-18) production after exposure to p-Phenylenediamine (PPD) in association with DBP. On the contrary, increased upregulation of CD86 and interleukin 8 (IL-8) production were observed in THP-1 cells exposed to combinations of citral (Cit) or imidazolidinyl urea (IMZ) with DBP, indicative of an adjuvant effect. Additionally, higher production of reactive oxygen species (ROS) in THP-1 cells treated with DBP associated to Cit supports that oxidative stress could be part of the molecular mechanism of the observed adjuvant effect. In conclusion, we demonstrate that DBP presents in vitro an adjuvant effect for immune stimulation in dendritic cells but not in keratinocytes. Future studies are necessary to elucidate the precise mechanism underlying the adjuvant effect of DBP in vitro and in vivo.

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1. Introduction

Phthalate esters are widely used as plasticizers in a variety of products, including soft polyvinyl chloride toys, vinyl floorings, wall coverings, synthetic leather and blood transfusion bags. Due to their wide distribution in the environment, they are considered agents with high potential for human exposure (Koo and Lee, 2004; Anderson et al., 2001; Silva et al., 2004; Lyche et al., 2009; Jeong et al., 2011). Some phthalate esters with short alkyl chains, like dibutyl phthalate (DBP), are used in cosmetics and mosquito repellents for topical use (Int. J. Toxicol., 2005; Vartak et al., 1994). Besides, phthalates with lower molecular weights are more highly absorbed (Elsisi et al., 1989).

DBP, as well as certain other phthalates, has attracted great attention from the scientific community because of their possible endocrine disruptive effects that can alter the normal development of the male reproductive tract (Fisher et al., 2003; Heudorf et al., 2007; Martino-Andrade et al., 2009). Besides the reproductive and endocrine effects, experimental and epidemiological data also suggest that phthalates could play a role as a possible contributor in the development and/or enhancement of atopic allergic diseases, thus, possibly contributing to the increased

prevalence of atopic IgE-mediated allergic diseases in industrialized countries (Imai et al., 2006; Koike et al., 2010; Matsuda et al., 2010; Li et al., 2014; Bornehag et al., 2004; Jaakkola et al., 2004). It has been suggested that certain phthalates may act as adjuvants, meaning that they could accelerate, prolong or enhance a specific immune response (Kimber and Dearman, 2010). However, no consistent pattern regarding to dose and route of exposure has emerged. Additional research is needed to better understand whether, to what extent, and under what conditions phthalates are able to effect a meaningful change in immune function that may in turn impact human health (Kimber and Dearman, 2010).

Allergic contact dermatitis is a delayed type hypersensitivity reaction resulting from repeated contacts of the skin with low molecular weight chemicals called haptens (Lepoittevin and Leblond, 1997; Saint-Mezard et al., 2004). Several cell types are involved in the contact hypersensitivity response. Keratinocytes respond to allergens by producing a range of pro-inflammatory cytokines, which are important for dendritic cell maturation and migration to the lymph node, where the activation of the specific immune response takes place (Vandebriel et al., 2005). Dendritic cells are recognized as important antigen presenting cells in adaptive immunity because of their capacity to stimulate naive lymphocytes (Banchereau et al., 2000).

Corsini et al. (2009) proposed an in vitro assay based on the interleukin 18 (IL-18) production in the human keratinocyte cell line NCTC 2544 to identify contact sensitizers and discriminate them from irritants and respiratory allergens (Galbiati et al., 2011; Corsini et al., 2013a). IL-18

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has been demonstrated to favor Th-1 type immune response by enhancing the secretion of pro-inflammatory mediators such as TNF- α , IL-8 and IFN- γ , and to play a key proximal role in the induction of allergic contact dermatitis (Cumberbatch et al., 2001). Mitjans et al. (2008) reported in the human promyelocytic cell line THP-1 (used as a surrogate of dendritic cells), a selective production of the chemokine interleukin 8 (IL-8) by contact allergens in parallel to the surface marker cluster of differentiation 86 (CD86) upregulation, and, proposed this as a possible in vitro sensitization test. IL-8 is a potent attractant for neutrophils and different subtypes of T cells (Barker et al., 1991).

Oxidative stress has been reported to be central to chemical allergen-induced contact dermatitis and inflammatory skin diseases (Okayama, 2005; Byamba et al., 2010; Corsini et al., 2013b). Several authors have reported a relationship between phthalates and oxidative stress demonstrating increasing generation of reactive oxygen species (ROS) (Seo et al., 2004; Tetz et al., 2013). Others have associated the presence of several phthalate monoester metabolites detected in urine, with increased serum markers of inflammation and oxidative stress (Hauser et al., 2007; Ferguson et al., 2011).

With this study, we aimed to investigate the mechanism underlying the possible adjuvant effect of DBP. We evaluated the effects of DBP alone or in association with selected chemical allergens in two in vitro models as representative of keratinocytes and dendritic cells responses, the human keratinocyte cell line NCTC 2544 and the human promyelocytic cell line THP-1, respectively. We also investigated if the generation of ROS could be part of the molecular mechanism of the adjuvant effect induced by DBP. Even though several studies have been conducted in animals to evaluate the adjuvant effect of phthalates, little can be found about this effect in vitro.

2. Material and methods

2.1. Chemicals

p-Phenylenediamine (PPD), citral (Cit) and imidazolidinyl urea (IMZ) were selected as skin sensitizers. Allergens and dibutyl phthalate (DBP) were purchased from Sigma (St. Louis, MO, USA) at the highest purity available (PPD 99%, Cit 95%, IMZ 95%, DBP 99%). PPD, Cit, IMZ and DBP were dissolved in DMSO (final concentration of DMSO in culture medium <0.2%). Lipopolysaccharide (LPS) from *Escherichia coli* serotype 0127: B8 was obtained from Sigma and stock solution was dissolved in PBS. LPS (10 ng/mL) was used as positive control in all the experiments with THP-1 cells and DMSO was used as vehicle control for experiments with both cell lines, THP-1 dendritic cells and NCTC 2544 keratinocytes. PPD was selected for keratinocyte activation, while Cit and IMZ were used for activation of dendritic cells based on previous experiments for the consistency and robustness of results. The doses of DBP were first selected according to the literature (Martinasso et al., 2006). The doses of the chemical sensitizers were chosen from previous experiments in the laboratory with slight changes after cell viability tests.

2.2. Cells

For experiments using NCTC 2544 cell line (Istituto Zooprofilattico di Brescia, Italy), cells were cultured in 24 or 96-well plates (0.5 or 0.1 mL/well, respectively) at density of 2.5×10^5 cells/mL. Cells were treated in 0.5 or 0.1 mL of RPMI 1640 containing 2 mM L-glutamine, 0.1 mg/mL streptomycin, 100 IU/mL penicillin, supplemented with 10% heated-inactivated fetal calf serum (media) and cultured at 37 °C in 5% CO₂.

For experiments using THP-1 cells (Istituto Zooprofilattico di Brescia, Italy), cells were used at a density of 10^6 cells/mL in RPMI

1640 containing 2 mM L-glutamine, 0.1 mg/mL streptomycin, 100 IU/mL penicillin, 50 μ M 2-mercaptoethanol, supplemented with 10% heated-inactivated fetal calf serum (media) and cultured at 37 °C in 5% CO₂. For cytotoxicity, IL-8 release, or CD86 expression, 0.5×10^6 cells were seeded in 24-well plates. For IL-8 mRNA expression 3.0×10^6 cells were seeded in 14 mL round bottom tubes.

2.3. Cell viability

NCTC cells (2.5×10^5 /mL) were seeded in a 96-well plate (0.1 mL/well) and treated with PPD alone or in association with DBP, or DMSO as vehicle control. After an incubation period of 24 h, culture media was discarded and 100 μ L/well of MTT solution 7.5 mg/mL in culture media was added. Cells were incubated for 3 h at 37 °C, media discarded and cells lysed in 100 μ L/well of a mixture of HCl 1 N: isopropanol (1:24). The absorbance of the resulting solutions was read at a wavelength of 595 nm in a microplate reader (Molecular Devices). Based on cell viability, cells were treated using the concentration of chemicals resulting in a cell viability >80%.

For THP-1 cells, cytotoxicity was assessed by flow cytometric evaluation of propidium iodide (PI)-stained cells. Cells were seeded in 24-well plates (0.5 mL/well) and incubated for 24 h with Cit or IMZ alone or in association with DBP, LPS as positive control, or DMSO as vehicle control. After incubation, cells were centrifuged at 1500 rpm for 5 min and suspended in 500 μ L PBS containing 1 μ g/mL PI. The percentage of positive cells was analyzed using a FACSCalibur flow cytometer, and data were quantified using CellQuest software (BD, Becton Dickinson). Based on cell viability, cells were treated using the concentration of chemicals resulting in cell viability >75%.

2.4. Cytokine production

For IL-18 production, NCTC cells were incubated with PPD (30 μ g/mL) alone or in association with DBP, or with DMSO as vehicle control. After incubation, culture media was discarded, monolayers gently washed once with 1 mL of PBS and cells lysed in 0.25 mL of 0.5% Triton X-100 in PBS. Plates were stored at –80 °C until measurement. Intracellular IL-18 content was assessed by specific sandwich ELISA commercially available (MBL, Nagoya, Japan). Results are expressed in pg/mg of total intracellular protein content. Limit of detection is 15.6 pg/mL. The protein content of the cell lysate was determined by the BCA method.

IL-8 release was measured in THP-1 cells after 24 h of incubation in 24-well plates (0.5 mL/well) with Cit or IMZ alone or in association with DBP, LPS as positive control, or DMSO as vehicle control. Cell-free supernatants were obtained by centrifugation at 1500 rpm for 5 min and stored at –20 °C until measurement. IL-8 was assessed by a specific sandwich ELISA commercially available (BD, Becton Dickinson). Results are expressed as pg/mL. Limit of detection is 15.6 pg/mL.

2.5. Flow cytometric analysis of CD86 expression

CD86 expression was evaluated by flow cytometric analysis. THP-1 cells were treated with the selected chemicals and associations, LPS or DMSO as vehicle control. After 24 h, cells were centrifuged at 1500 rpm for 5 min. Supernatants were collected for IL-8 assessment, and cells were stained for 30 min with a specific FITC-conjugated antibody against CD86 (BD, Becton Dickinson) or with isotype control antibody at 4 °C following supplier's instructions. The intensity of fluorescence and the percentage of positive cells were analyzed using FACSCalibur flow cytometer, and data were quantified using CellQuest software (BD, Becton Dickinson). 10,000 viable cells were analyzed for mean fluorescence intensity (MFI) and percentage of positively labeled cells (PC). Changes in

CD86 expression are reported according to Azam et al. (2006) as stimulation index (SI) calculated by the following equation:

$$SI = \frac{PC_t \times MFI_t}{PC_c \times MFI_c}$$

PC_c and MFI_c stand for the percentage of positive cells and mean fluorescence intensity in control cells, whereas PC_t and MFI_t stand for the percentage and mean fluorescence intensity in chemical-treated cells.

2.6. Real Time RT-PCR

Total RNA was isolated from THP-1 cells after 3 h of treatment with selected chemicals and associations using commercial available kit (TriReagent from Sigma) as described by the manufacturer. For the synthesis of cDNA, 2.0 µg of total RNA was retro-transcribed using a high-capacity cDNA archive kit from Applied Biosystems (Foster City, CA, USA) following the supplier's instructions. IL-8 gene expression was evaluated by Real Time reverse transcription polymerase chain reaction (Real Time PCR). For PCR-analysis, Taq-Man™-PCR technology was used. For each PCR reaction, 10 ng of total RNA were used. The 18S ribosomal RNA transcription was used as endogenous reference and the quantification of the transcripts was performed by the $\Delta\Delta C_T$ method.

2.7. Determination of intracellular reactive oxygen species

For determination of intracellular reactive oxygen species (ROS), the fluorescent intensity of dichlorofluorescein diacetate (DCFH-DA) (Molecular Probes, Eugene, OR, USA) oxidized product, DCF, was measured. THP-1 cells (1 mL in round bottom cytometer tubes) were loaded with DCFH-DA (2 µM) for 15 min and kept away from light. After fixation, cells were analyzed with FACScan flow cytometry at time 0, and then treated with DBP (500 µM/mL), Cit (15 µg/mL), Cit + DBP, LPS (10 ng/mL) as positive control, or DMSO as vehicle control. Cells were then acquired at different time points: 5, 15, 30, and 60 min. The fluorescence intensity of fluorophore DCF, which was formed by peroxide oxidation of the non-fluorescent precursor, was measured (in the FL1 channel set for log scale) using FACSCalibur flow cytometer, and data were quantified using CellQuest software (BD, Becton Dickinson).

2.8. Data analysis

All experiments were performed at least two times, with representative results shown. The data presented are expressed as mean $\pm$ SD. Statistical significance was determined by ANOVA followed by Tukey multiple comparison test using the GraphPad Prism software (GraphPad Software, La Jolla, CA). The comparison of IMZ with negative control (DMSO) in the analysis of mRNA was performed by unpaired t test with Welch's correction. Differences were considered significant for $p \leq 0.05$.

3. Results

3.1. Effect of the selected chemicals on cell viability

The chemical concentrations resulting in 80% of viability in relation to vehicle treated NCTC cells 24 h after treatment were calculated for PPD and the association of this chemical with three doses of DBP. Based on these results, a dose of 30 µg/mL of PPD and 1000 µM of DBP were selected. Two lower doses of DBP (500 and 250 µM) were selected to evaluate a dose response of the adjuvant effect of this substance.

THP-1 cells were treated with increasing concentrations of the selected chemicals and associations for 24 h. Cell viability was then assessed by flow cytometry using PI staining. According to the results, based on cell viability, cells were then treated with concentrations of

chemicals resulting in more than 75% of viability, which corresponded to 15 µg/mL of Cit and 30 µg/mL of IMZ. DBP was used in the same doses as with NCTC.

3.2. Effects of the selected chemicals on the release of IL-18

NCTC cells were treated for 24 h with PPD alone or in association with one of the three doses of DBP. PPD induced an increase in intracellular IL-18 content, but there was no higher increase in the groups exposed to the association between PPD and any of the doses of DBP. DBP alone did not induce the release of IL-18 (data not shown).

3.3. Effects of the selected chemicals on the release of IL-8

THP-1 cells were first treated for 24 h with the selected concentrations of DBP. None of the doses of DBP were able to induce the release of IL-8 (data not shown).

Cells were then treated with Cit or IMZ, or the associations between these chemicals and the increasing doses of DBP. As shown in Fig. 1, Cit induced the release of IL-8, and, the three doses of DBP in association significantly increased this release. Similar results were obtained when IMZ was associated with increasing doses of DBP. All doses of DBP were able to increase the IL-8 release induced by IMZ (Fig. 1).

3.4. CD86 expression after citral, IMZ and DBP exposure

In parallel to IL-8 release, the effect of the selected chemicals and associations on CD86 expression was investigated. Similar to the results obtained with IL-8 release, exposure to Cit showed an increase in the expression of CD86, which was significantly higher in the groups exposed to the associations between Cit with DBP (Fig. 2). IMZ was also able to increase CD86 expression and higher expressions were observed when IMZ was associated with the doses of 500 and 1000 µM of DBP, but not with 250 µM (Fig. 2). DBP alone did not induce the expression of CD86 in any of the doses (data not shown).

3.5. Effects on IL-8 mRNA expression

Since IL-8 seems to be a more sensible marker on distinguishing sensitizers, we investigated the expression of the IL-8 mRNA to investigate if the adjuvant effect of DBP in the release of IL-8 occurred before or after the transcription of IL-8 mRNA. THP-1 cells exposed to Cit showed IL-8 mRNA expression as assessed by Real Time PCR. IMZ induced a 2.7 fold increase in the IL-8 mRNA expression when compared with DMSO. When ANOVA was performed to analyze all the groups for this parameter (DMSO, IMZ, DBP and IMZ associated to DBP), we could not identify a significant difference between IMZ and DMSO because the increase in the association was too high and overshadowed this result. However, when we compared only DMSO with IMZ in a t test, the significant difference was apparent. Both Cit and IMZ, when associated with 500 µM of DBP, presented a significant increase in mRNA expression (Fig. 3). DBP, which tested negative for the release of IL-8 after 24 h of treatment, was able to induce the mRNA expression after 3 h of incubation (Fig. 3).

3.6. Effects on intracellular reactive oxygen species (ROS)

Fig. 4 shows the ability of the selected chemicals to generate reactive oxygen species assessed by the DCFH-DA assay in THP-1 cells. The exposure to Cit increased DCFH-detected ROS levels at 5 min of treatment, which was maintained until the end of the evaluation (60 min). This increase was enhanced when Cit was associated with 500 µM of DBP in all evaluated time points. DBP alone also induced a slight increase in ROS, and this was more evident at 5 min of treatment.

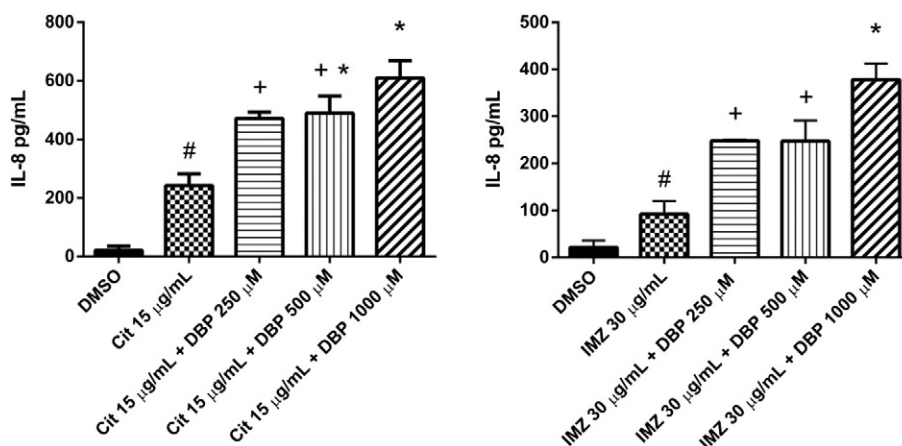


Fig. 1. Effects of the selected chemicals and associations on IL-8 release. THP-1 cells were treated for 24 h with citral (Cit), imidazolidinyl urea (IMZ) or the associations between Cit or IMZ with increasing doses of dibutyl phthalate (DBP). Vehicle treated cells were included as a vehicle control (DMSO). IL-8 release was measured by ELISA in culture supernatants, results are expressed as pg/mL. Each value represents the mean $\pm$ SD, $n = 3$. Different symbols over the bars represent significant statistical difference. $p < 0.05$, ANOVA followed by Tukey multiple comparison test.

4. Discussion

It has been postulated that phthalate esters may be associated with facilitation in the development of allergic diseases such as asthma and atopic dermatitis (Bornehag et al., 2004; Jaakkola et al., 2004). Additionally, certain phthalates have been shown to enhance the response to allergens in animal models of allergic contact dermatitis (Imai et al., 2006; Li et al., 2014). The underlying mechanisms, however, have not been fully elucidated. The present in vitro study investigated the effects of the plasticizer DBP on chemical allergen-induced cell activation. In particular, NCTC 2544 and IL-18 production were used as surrogate of contact allergen-induced keratinocyte activation, while the upregulation of CD86 and IL-8 production in THP-1 cells were used as markers of dendritic cell (DC) activation. Results obtained suggest an adjuvant effect of DBP in DC but not in epidermal keratinocytes, as demonstrated by the lack of co-stimulation in PPD-induced IL-18 production.

Since humans are commonly exposed to phthalates, it is important to elucidate potential immunotoxic effects of the same. With the widespread use of DBP, its potential toxicity is getting increasing attention. It is known that phthalate esters lack the ability to bind to proteins and form a hapten–protein complex, which is required for the stimulation

of allergic sensitization (Kimber and Dearman, 2010). According to epidemiological data, phthalate esters do not have the potential to cause dermal irritation or skin sensitization (Medeiros et al., 1999; Geier et al., 2004). However, even though evidence supports a lack of sensitizing potential, it has been suggested that certain phthalates may act as adjuvants by enhancing immune responses.

The NCTC 2544 IL-18 assay is a useful in vitro tool that helps identify contact sensitizers and discriminate them from irritants and respiratory allergens (Corsini et al., 2009; Galbiati et al., 2011; Corsini et al., 2013a). In this study, as expected, the contact allergen PPD induced a statistically significant increase in the production of IL-18, while DBP alone failed to induce it, supporting the evidence that phthalates lack the potential to cause sensitization. The association with DBP failed, however, to further increase IL-18 production, suggesting that DBP does not act as an adjuvant in keratinocytes.

Mitjans et al. (2008) demonstrated that chemical allergens induce a dose-related release of interleukin-8 as well as the expression of the surface marker CD86 in the human promyelocytic cell line THP-1, proposing this as an in vitro assay for the screening of potential chemical allergens. In this work, the CD86 upregulation as well as IL-8 release were investigated after exposure of THP-1 cells to two contact allergens, citral and imidazolidinyl urea, and their associations with DBP. According to the local lymph node assay (LLNA), citral is classified as moderate

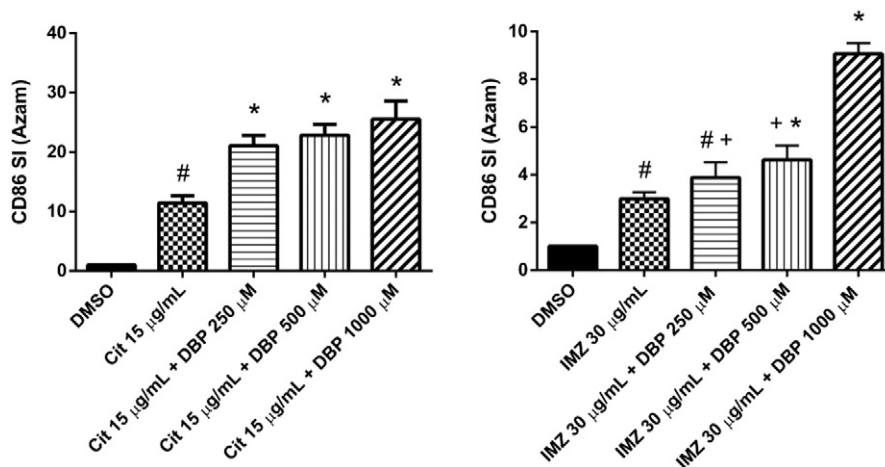


Fig. 2. Effects of the selected chemicals and associations on the expression of cell surface marker CD86. THP-1 cells were treated for 24 h with citral (Cit), imidazolidinyl urea (IMZ) or the associations between Cit or IMZ with increasing doses of dibutyl phthalate (DBP). Vehicle treated cells were included as a vehicle control (DMSO). Results are expressed as stimulation index (SI). Each value represents the mean $\pm$ SD, $n = 3$. Different symbols over the bars represent significant statistical difference. $p < 0.05$, ANOVA followed by Tukey multiple comparison test.

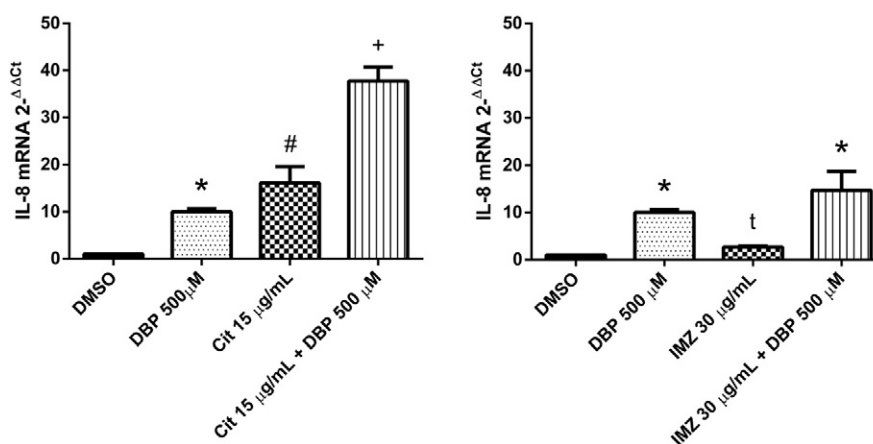


Fig. 3. Effects of the selected chemicals and associations on IL-8 mRNA expression. THP-1 cells were treated for 3 h with citral (Cit), imidazolidinyl urea (IMZ), dibutyl phthalate (DBP) or the associations between Cit or IMZ with increasing doses of DBP. Vehicle treated cells were included as a vehicle control (DMSO). IL-8 mRNA expression was evaluated by Real Time-PCR as described in the [Material and methods](#) section. Results are expressed as $2^{-\Delta\Delta C_t}$. Each value represents the mean $\pm$ SD, $n = 3$ independent experiments. Different symbols over the bars represent significant statistical difference. $p < 0.05$, ANOVA followed by Tukey multiple comparison test. (t) Significant difference when compared with DMSO. $p < 0.05$, Unpaired t test with Welch's correction.

sensitizer with an EC₃ value of 9.3%, while imidazolidinyl urea is classified as a weak sensitizer with an EC₃ value of 24%. Both allergens induced a statistically significant upregulation in the expression of CD86. DBP alone did not induce this expression, further supporting the lack of sensitizing potential. In accordance with our data, [Ito et al. \(2012\)](#),

showed that di (2-ethylhexyl) phthalate (DEHP) did not induce the expression of dendritic cells differentiation and maturation markers, including CD86, in mouse peripheral blood mononuclear-derived dendritic cells. On the contrary, [Koike et al. \(2009,2010\)](#) using bone marrow-derived dendritic cells obtained from atopic prone NC/Nga mice,

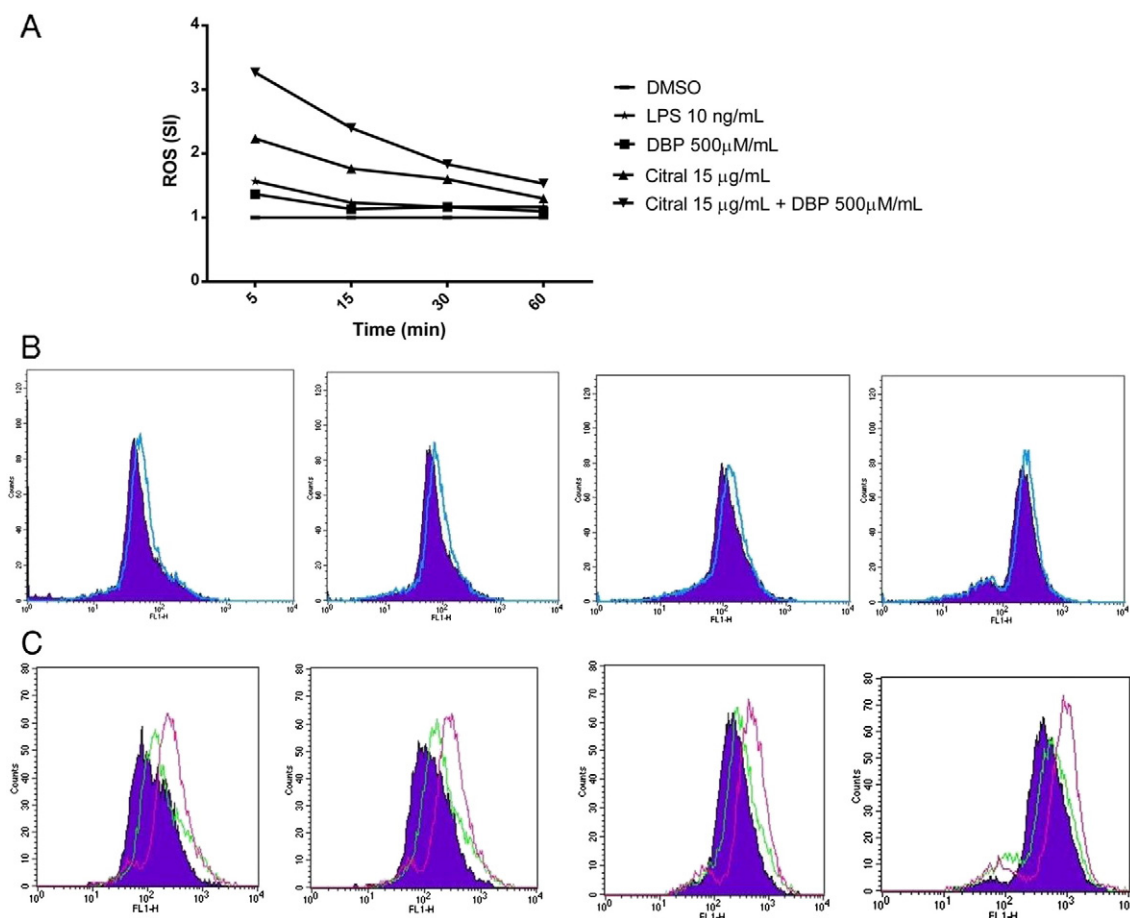


Fig. 4. Effects of the selected chemicals and associations on intracellular reactive oxygen species. THP-1 cells were loaded with DCFH-DA and then treated with dibutyl phthalate (DBP, 500 μM), citral (Cit, 15 μg/mL), Cit + DBP, LPS (10 ng/mL) as positive control, or DMSO as vehicle control. Cells were then acquired at different time points: 5, 15, 30, and 60 min. The fluorescence intensity of fluorophore DCF was measured by flow cytometry as described in the [Material and methods](#) section. Results are expressed as stimulation index (SI). (A) Each value represents the mean, $n = 3$ independent experiments. (B) THP-1 cells were treated with DBP 500 μM (blue) for 5, 15, 30 and 60 min. The purple histogram represents control cells. (C) THP-1 cells were treated with Cit 15 μg/mL (green) or Cit associated with DBP 500 μM (pink) for 5, 15, 30 and 60 min. The purple histogram represents control cells.

found that DEHP as well as diisononyl phthalate (DINP) enhanced the expression of CD86 and other costimulatory molecules. Different culture conditions, cells or experimental design could explain these differences.

The exposure of THP-1 cells to Cit and IMZ revealed a statistically significant release of IL-8 compared to vehicle treated cells. Again, DBP alone failed to induce the release of IL-8. Data on phthalate-induced release of cytokines in vitro is conflicting. Although it has been reported that some phthalate monoesters, including the DBP metabolite monobutyl phthalate (MPB), do not induce the release of cytokines after exposure in THP-1 cells (Glue et al., 2002), others have shown that phthalate monoesters lead to the release of IL-8 in the epithelial cell line A549 (Jepsen et al., 2004). In the study by Jepsen et al. (2004), high doses of MBP induced only a slight increase in IL-8 release, and more significant increases were obtained with the exposure to monoesters with more than eight carbon atoms in their alkyl side chain. According to their results, these authors suggest that phthalate monoesters with fewer than eight carbon atoms in their alkyl side chain (like MBP) are weak cytokine inducers, whereas monoesters with eight or more carbon atoms in the alkyl side chain are more potent cytokine inducers.

When THP-1 cells were exposed to either Cit or IMZ associated with DBP, cells exhibited higher expression of CD86 and release of IL-8, when compared to the exposure to the allergen alone. This indicates an adjuvant effect of DBP on THP-1 cells. Even though several studies have been performed to evaluate the effects of phthalates in cell function, little can be found in the literature regarding the adjuvant effect of these substances in vitro. Nevertheless, a great amount of studies have been performed about the adjuvant effect of phthalates in vivo. Several authors have showed that different phthalates can act as adjuvants in contact hypersensitivity and airway inflammation, elevating the expression of surface markers and related cytokines after exposure to associations between allergens and phthalates by different protocols (Imai et al., 2006; Koike et al., 2010; Matsuda et al., 2010; Li et al., 2014). Corroborating our results, Tanaka et al. (2012) demonstrated that DEHP exacerbated ovalbumin-related peritoneal inflammation characterized by enhanced infiltration of macrophages and neutrophils and amplified production/release of cytokines such as keratinocyte-derived chemoattractant (KC) in the peritoneal cavity. KC is a murine homolog of IL-8 (Hol et al., 2010). Furthermore, Takano et al. (2006) showed that exposure to DEHP in the presence of allergen increased the expression of MIP-1 α compared to vehicle combined with allergen only. MIP-1 α functions as chemotactic for neutrophils similarly to IL-8 (Hammond et al., 1995; Menten et al., 2002).

According to Galbiati et al. (2012) the expression of IL-8 mRNA at 3 h is important since some chemical allergens fail to induce the release of IL-8 after 24 h of exposure, but all chemical sensitizers tested induced IL-8 mRNA at 3 h. Both Cit and IMZ induced the expression of IL-8 mRNA, and this expression was significantly higher when associated with 500 μ M of DBP. As IMZ is a weak sensitizer, its induction of the expression of IL-8 mRNA was lower compared to the induction following Cit exposure. IMZ induced a 2.7 fold increase in IL-8 mRNA, still significantly different from the control group (Welch's t test). Interestingly, DBP, which failed to induce the expression of the surface marker CD86 or the release of IL-8, induced a significant expression of IL-8 mRNA. As mentioned before, data about the release of pro-inflammatory cytokines, including IL-8, induced by phthalates is conflicting. While some authors found that certain monophthalates can induce this release at high doses, others report no release after monophthalates exposure (Glue et al., 2002; Jepsen et al., 2004). Overall, little can be found in literature relating DBP exposure and increase in pro-inflammatory cytokines mRNA expression. The control of translation and mRNA degradation is an important part of the regulation of gene expression. MicroRNAs (miRNAs) are a class of small RNA molecules that modulate gene expression by interacting with the messenger RNA of the respective target gene (Schneider, 2012). miRNAs can silence mRNAs either

by triggering endonuclease cleavage, by promoting translation repression, or by accelerating mRNA decapping (Valencia-Sanchez et al., 2006). It's been shown that certain miRNAs are upregulated in allergic contact dermatitis (Vennegaard et al., 2012) and other skin inflammatory diseases (Sonkoly et al., 2010; Zibert et al., 2010), although still scarce information can be found about the function of miRNAs in skin sensitization. Since the silencing of mRNAs by miRNAs occurs through a very specific interaction between the miRNA and its targeted mRNA (Valencia-Sanchez et al., 2006), this could be a possible explanation to why only the IL-8 mRNA upregulated by DBP was not translated into protein. However, this is something that needs further investigation.

Although previous in vivo studies have shown that certain phthalates aggravate allergic response and/or diseases (even though in doses considerably higher than those relevant for human exposure), the underlying cellular and molecular mechanisms remain unclear. Evidence indicates that allergic and inflammatory skin diseases could be mediated by oxidative stress (Okayama, 2005; Byamba et al., 2010). Mizuashi et al. (2005) hypothesized that sensitizers induce oxidative stress in dendritic cells, which subsequently stimulates p38 MAPK. Their results demonstrated that sensitizers reduced the oxidized (GSSG) versus reduced (GSH) form of cellular glutathione (GSH/GSSG ratio) in THP-1 cells and phosphorylated p38 MAPK. According to Mitjans et al. (2008), the activation of the p38 MAPK by sensitizers plays a crucial role in the activation of IL-8 release. In this study, together with increase in IL-8 release, we showed a slight increase of ROS generation induced by DBP alone, but, more importantly, a significant increase in the oxidative stress induced by DBP when in association with Cit. Others have demonstrated that some phthalates can induce oxidative stress. Tetz et al. (2013) reported an increase in ROS production induced by MEHP in human placental cells. DEHP also increased the generation of ROS in testis inducing apoptosis of spermatocytes (Kasahara et al., 2002). Moreover, You et al. (2014) showed significantly higher levels of ROS in ovalbumin sensitized mice that were co-treated with DEHP when compared to the levels of ROS of the sensitized-only group. These authors suggest that oxidative stress plays a critical role in the adjuvant effect of DEHP.

In the present study we demonstrated a positive adjuvant effect of DBP when associated with citral and imidazolidinyl urea as indicated by the increased expression of CD86, IL-8 mRNA and IL-8 release. We also suggest that the increased generation of ROS could be the trigger that leads to these effects. On the contrary, we found no adjuvant effect on keratinocytes. Taken together, our results support that the adjuvant effect of DBP relies on dendritic cells. Previously published data corroborate our results. Koike et al. (2009) and Ito et al. (2012), based on their in vivo and in vitro studies, suggest that DEHP accelerates murine dermatitis through the inappropriate activation of dendritic cells. Koike et al. (2010) proposed that DINP may activate dendritic cells in inflammatory sites through the enhancement of thymic stromal lymphopoietin (TSLP) production. The production of TSLP has also been shown to be induced by DBP (Larson et al., 2010). Imai et al. (2006) suggested that DBP and DPP facilitate the trafficking of Langerhans cells and/or myeloid dendritic cells from skin sites in contact hypersensitivity induced by fluorescein isothiocyanate.

In an immunological context an adjuvant is a material that is able to enhance immune responsiveness without itself necessarily providing any specific antigenic stimulus (Kimber and Dearman, 2010). From the results on CD86 expression and IL-8 release, where DBP alone had no effects, an enhanced upregulation of both markers was observed when DBP was associated to Cit or IMZ, suggesting that DBP acts as an adjuvant by immunological definition. Since evidence that phthalates can act as sensitizers themselves is scarce and as other authors have suggested in different animal models of dermatitis that phthalates act as adjuvants, we believe that the effects found in our work are consistent with an adjuvant effect. Interestingly, this effect seems to be specific for immune cells as no additive effects were observed in chemical

allergen-induced IL-18 production in keratinocytes in the presence of DBP.

In conclusion, we propose that DBP presents in vitro an adjuvant effect when associated with sensitizers. However, the effects of DBP in vivo remain incompletely understood, thus, future studies are necessary to elucidate the precise mechanism of adjuvant effect of DBP not only in vitro but also in vivo.

Conflict of interest

The authors declare no conflicts of interest.

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