Activation of the Endoperoxide Ascaridole Modulates Its Sensitizing Capacity

Nora L. Krutz,* Jennifer Hennen,* Corinna Korb,* Mario T. Schellenberger,* G. Frank Gerberick,† and Brunhilde Blömeke*,†

*Department of Environmental Toxicology, University Trier, 54296 Trier, Germany and †Central Product Safety Department, The Procter & Gamble Company, Mason Business Center, 8700 Mason Montgomery Road, Cincinnati, OH

ABSTRACT

The monoterpene ascaridole, a fairly stable endoperoxide found in essential oils such as tea tree oil can provoke allergic contact dermatitis which has been evidenced under patch test conditions. However, concomitantly we observed irritative skin reactions that demand further data underlining the sensitization potential of ascaridole. Here, we studied the effects of ascaridole on dendritic cell (DC) activation and protein reactivity, 2 key steps of chemical-induced skin sensitization. Treatment of human monocyte-derived DC with ascaridole found support for full DC maturation, a capability of sensitizers but not irritants. It induced significant upregulation of the expression of the costimulatory molecules CD86, CD80, CD40, and the adhesion molecule CD54 in a time-dependent manner. Maturation was accompanied by release of proinflammatory cytokines interleukin (IL)-1ß, tumor necrosis factor-α, IL-6, and IL-8. Similar to other chemical skin sensitizers including hydroperoxides, we observed a certain reactivity of ascaridole toward cysteine- but not lysine-containing peptides. During recent years, evidence accumulated for a radical mechanism as trigger for protein reactivity of peroxides. Treatment of the fairly stable endoperoxide ascaridole with iron as radical inducer (“activated ascaridole”) resulted in cysteine peptide reactivity exceeding by far that of ascaridole itself. Furthermore, activated ascaridole showed increased potential for induction of the Nrf2 target gene heme oxygenase 1 and upregulation of CD86 and CD54 on THP-1 cells, an established DC surrogate. These results indicate that radical formation could be involved in the steps leading to skin sensitization induced by the endoperoxide ascaridole.

Key words: ascaridole; endoperoxide; terpene; monocyte-derived dendritic cells (MoDC); THP-1; skin sensitization

Chemical-induced allergic contact dermatitis (ACD) is one of the most common occupational and environmental issues of industrial societies. The most common allergens in cosmetic-related products causing ACD are fragrances and preservatives commonly used in household and cosmetic products (Alani et al., 2013; SCCS, 2012). ACD to cosmetic-related allergens has increased over the years and affects about 1% of the general population (Park and Zippin, 2014). Some investigators suspect that ingredients of botanicals or essential oils of certain plants that are increasingly used as antioxidants in consumer products (eg, shaving oil, soap, and facial masks), can be activated themselves and thereby form new sensitizers, and are at least partially involved in this increasing trend (Jack et al., 2013).

In this context, consumer products containing in particular tea tree oil (TTO) gained increased popularity. Among many others, ascaridole (1,4-epidioxy-2-p-menthene, Fig. 1) was inconsistently detected in TTO (eg, Rudbäck et al., 2012; Sciarrone et al., 2010) and also identified as an oxidation product of limonene (Pokorny et al., 1998). On this background, the elicitation potential of ascaridole in individuals with eczema and suspected fragrance allergy after topical exposure was studied in the past (Hausen, 2004) and more recently by our team (Bakker et al., 2011; Christoffers et al., 2013, 2014). Application of 1% and 2% ascaridole in petrolatum under diagnostic patch test conditions revealed positive reactions in 1.4% and 5.5% among 290 tested individuals, respectively (Christoffers et al., 2014).
However, we also observed irritant reactions (in 5/290 patients), whose occurrence also augmented with increasing concentrations. This observed concomitant irritancy still causes debates regarding the human sensitization potential of ascaridole.

According to the Adverse Outcome Pathway (AOP) for Skin Sensitisation (OECD, 2012) additional experimental support for being a chemical skin sensitizer derives from analysis of key events such as covalent binding to proteins and the ability to induce signaling processes involved in the activation of dendritic cells (DC) to mature and migrate to the draining lymph node for interaction with T cells (Kaplan et al., 2012). Monocyte-derived DC (MoDC) are commonly used to demonstrate cellular changes including maturation and cytokine release induced by chemicals (Alba et al., 1997; Tuschl and Kovac, 2001). In addition, a number of investigations (Bocchiotto et al., 2007; Yoshiida et al., 2003) including ours (Hennen et al., 2011; Tietze and Blömeke, 2008) have demonstrated that CD86 and CD54 surface expression on THP-1 cells can be used as markers for DC activation.

For a number of terpenes and hydroperoxides, studies found a gain of protein reactivity under radical formation conditions including maturation and cytokine release induced by chemicals (Alba et al., 1997; Tuschl and Kovac, 2001). In addition, a number of investigations (Bocchiotto et al., 2007; Yoshiida et al., 2003) including ours (Hennen et al., 2011; Tietze and Blömeke, 2008) have demonstrated that CD86 and CD54 surface expression on THP-1 cells can be used as markers for DC activation.

Cell culture. Peripheral blood mononuclear cells were isolated from buffy coats of healthy donors using Ficoll density gradient centrifugation. Monocytes were isolated and purified as described (Hauser et al., 1994), and subsequently used for generation of MoDC. MoDC were cultured in RPMI 1640 supplemented with 10% FBS, 1% glutamine, antibiotic/antifungal solution, and PBS and were obtained from PAA (Co~lbe, Germany). Ficoll-Paque PLUS was obtained from GE Healthcare (Freiburg, Germany). Granulocyte–macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4 were from PeproTech (London, United Kingdom). RPMI 1640 medium, fetal bovine serum (FBS), l-glutamine, antibiotic/antimycotic solution, and PBS were obtained from PAA (Göteborg, Sweden). Ficoll-Paque PLUS was obtained from GE Healthcare (Freiburg, Germany). Granulocyte–macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4 were from PeproTech (London, United Kingdom). Random hexamers, RNase inhibitor, MuIV reverse transcriptase, PCR-buffer, magnesium chloride (MgCl2) and dNTPs were purchased from Applied Biosystems (Weiterstadt, Germany). DNA oligonucleotides were synthesized by Eurogentec (Cologne, Germany). High Pure RNA Isolation kit and the LightCycler-FastStart DNA Master SYBR Green I kit were purchased from Roche Diagnostics (Mannheim, Germany). IL-8 ELISA was purchased from R&D Systems (Wiesbaden, Germany). Cytometric Bead Array (CBA) Human Inflammation Kit was from BD Biosciences (Heidelberg, Germany). If not otherwise indicated, assays were always conducted according to the manufacturer’s instructions. Other reagents were obtained from Sigma-Aldrich (Taufkirchen, Germany) or Merck KGaA (Darmstadt, Germany).

FIG. 1. Chemical structure of ascaridole.
20 μM) served as established positive control for the upregulation of CD86 and CD54 on THP-1 cells as well as cysteine reactive sensitizer, and tBHQ (40 μM) was used as well-known Nrf2 activator. When indicated, ascaridole (50 mM) was preincubated with ferrous sulfate (FeSO₄, 10 mM) dissolved in PBS (1 h, room temperature, protected from light) and further referred to as activated ascaridole. Medium, DMSO (0.2%) and FeSO₄-treated cells (60–180 μM) served as corresponding controls.

Flow cytometry analysis of MoDC and THP-1 cells. Upon treatment for 24 h (THP-1, MoDC) and 96 h (MoDC), cells were harvested, washed with PBS, and incubated with anti-CD86-APC, anti-CD86-PE or anti-CD86-PerCP (clone 2331 (FUN-1)), anti-CD80-PerCP (clone L307.4), anti-CD40-PerC (clone SC3), anti-CD54-PE or anti-CD54-APC (clone HA58) and corresponding isotype controls (BD Biosciences, Heidelberg, Germany). PI-negative cells were excluded from the analysis. Results obtained with MoDC are shown as the mean fluorescence intensity (MFI) corrected by the corresponding isotype control. For elucidating the impact of the iron-treatment of ascaridole, results obtained in these experiments with THP-1 cells are given as the relative fluorescence intensity (RFI) (Sakaguchi et al., 2007) by relating the isotype-corrected MFI of chemical-treated cells to isotype-corrected MFI of corresponding controls.

Cytokine quantification in cell culture supernatants of MoDC. Culture supernatants were harvested after treatment of MoDC for 24 h and stored at −80 °C until further analysis. IL-1β (limit of detection: 7.2 pg/ml), tumor necrosis factor (TNF)-α (limit of detection: 7.5 pg/ml), and IL-6 (limit of detection: 2.5 pg/ml) were measured by CBA. IL-8 (limit of detection: 3.6 pg/ml) was measured by ELISA.

RNA isolation, reverse transcription and quantitative real-time PCR. For quantification of heme oxygenase 1 (HMOX1, NM_002133.2) mRNA levels, THP-1 cells were exposed to test chemicals for 6 h (Migdal et al., 2013). Cell viability was determined in pretests conducting the resazurin metabolism assay as described (Klein et al., 2013). According to that, test concentrations of ascaridole that reduced cell viability to up to 70% (data not shown) were chosen for subsequent mRNA analysis.

Total RNA was isolated with the High Pure RNA Isolation Kit. RNA was quantified and reverse transcribed (0.5 μg/μl) using a GeneAmp PCR System 9700 (Applied Biosystem Weiterstadt, Germany) as described previously (Kalnes and Blömeke, 2012).

The quantitative PCR was performed on a capillary LightCycler 2.0 instrument using LightCycler-FastStart DNA Master SYBR Green 1 Kit. Briefly, PCR reaction contained 25 ng cDNA, 2 mM MgCl₂, 1 μM forward and reverse primer, and 1 μl 10 × SYBR Green Fast-Start Master Mix in a final volume of 10 μl. Oligonucleotide sequences for HMOX1 were 5′-TTC TCC TGC GGG TCC TTA CAC T-3′ and 5′-GGC ATA AAG CCC TAC AGC AAC T-3′ (Rapozzo et al., 2009). Oligonucleotide sequences for the reference gene hypoxanthine phosphoribosyltransferase 1 (HPRT1; NM_000194) were 5′-TGA CAC TGG CAA AAC AAT GCA-3′ and 5′-GGT CCT TTT CAC CAG CAA GCT-3′ (Vandesompele et al., 2002). The temperature profiles included an initial denaturation step at 95 °C for 10 min followed by 40 amplification cycles with denaturation at 95 °C for 3 s, annealing at 60 °C for HMOX1 and 55 °C for HPRT1 for 3 s, and elongation at 72 °C for 3 s. The specificity of the PCR product was confirmed by melting curve analysis. Quantitative values were obtained from the threshold cycle (ct) and cDNA levels normalized to HPRT1 using 2⁻ΔΔct method (Livak and Schmittgen, 2001).

Peptide reactivity. Protein reactivity potential of ascaridole was evaluated as reactivity toward cysteine- or lysine-based synthetic peptides as previously described (Gerberick et al., 2004, 2009; Goebel et al., 2014). Briefly, reactivity was determined upon incubating 0.5 mM cysteine- and lysine-containing peptide with 5 mM and 25 mM ascaridole (dissolved in ethanol), respectively, for up to 24 h. Additionally, activated ascaridole was generated by incubating 20 mM ascaridole with 4 mM FeSO₄ dissolved in H₂O (resulting in a ratio concentration ascaridole-concentration FeSO₄ of 5:1) for 1 h at room temperature, protected from light, prior to addition of 0.5 mM cysteine peptide, yielding a final concentration of 5 mM preincubated ascaridole (25 mM in case of the lysine approach). Ethanol (5% and 25%) and FeSO₄ (1 mM and 5 mM) served as corresponding controls. In addition, 5 mM p-benzoquinone and DNCB served as positive/reference controls. After incubation, samples were analyzed by HPLC/PDA. Furthermore, enzyme-mediated modulation of reactivity of ascaridole (2.5 and 5 mM) toward the cysteine peptide (20 μM) was determined in samples additionally containing HRP and hydrogen peroxide (HRP/P) at 3 U/ml and 100 μM, respectively, and deferoxamine (10 μM). Experiments devoid of HRP/P were conducted in parallel. Eugenol (0.2 mM) and glutaraldehyde (5 mM) served as positive and reference controls. Following 24 h incubation, samples were processed and analyzed by HPLC/MS/MS. The peak area of cysteine model peptide was related to the internal standard. Peptide reactivity is given as percent peptide depletion, calculated by comparing the peak area of samples containing ascaridole to corresponding controls.

Statistical analysis. When applicable, differences between treated cells and corresponding controls were determined by using either a paired or unpaired t test on log-transformed data. A p value <.05 was considered to be statistically significant.

RESULTS

Induction of DC Maturation by Ascaridole

One hallmark of skin sensitizers is the induction of DC maturation. We therefore first focused on the phenotypic changes on MoDC (from up to 9 different donors) induced by ascaridole. Overall, augmentation of costimulatory molecules (CD86, CD40, and CD80) and/or the adhesion molecule CD54 was observed after a 24 h and/or 96 h incubation of MoDC with 60 μM ascaridole, presented as MFI for each marker (Fig. 2). On the individual level we observed time- and concentration-dependency but magnitude as well as cytotoxicity (percentage of PI-positive cells) varied between the donors. In the presence of this amount of ascaridole CD86 was significantly upregulated in every donor after 24 h (n = 6), and levels were even more augmented in 8 of 9 donors after 96 h (Fig. 2A). The cell surface expression of CD40 reached significance after 96 h (6 of 6 donors, Fig. 2B). Furthermore, CD80 was increased after 24 h in 1 of 6 donors but the effects were more pronounced after 96 h (7 of 9 donors, Fig. 2C). In addition, CD54 was clearly, although not reaching statistical significance, upregulated after 24 h (5 of 8 donors) and 96 h (4 of 5 donors, Fig. 2D). All donors presented responded to the positive control LPS. 

Peptide reactivity. Protein reactivity potential of ascaridole was evaluated as reactivity toward cysteine- or lysine-based synthetic peptides as previously described (Gerberick et al., 2004, 2009; Goebel et al., 2014). Briefly, reactivity was determined upon incubating 0.5 mM cysteine- and lysine-containing peptide with 5 mM and 25 mM ascaridole (dissolved in ethanol), respectively, for up to 24 h. Additionally, activated ascaridole was generated by incubating 20 mM ascaridole with 4 mM FeSO₄ dissolved in H₂O (resulting in a ratio concentration ascaridole-concentration FeSO₄ of 5:1) for 1 h at room temperature, protected from light, prior to addition of 0.5 mM cysteine peptide, yielding a final concentration of 5 mM preincubated ascaridole (25 mM in case of the lysine approach). Ethanol (5% and 25%) and FeSO₄ (1 mM and 5 mM) served as corresponding controls. In addition, 5 mM p-benzoquinone and DNCB served as positive/reference controls. After incubation, samples were analyzed by HPLC/PDA. Furthermore, enzyme-mediated modulation of reactivity of ascaridole (2.5 and 5 mM) toward the cysteine peptide (20 μM) was determined in samples additionally containing HRP and hydrogen peroxide (HRP/P) at 3 U/ml and 100 μM, respectively, and deferoxamine (10 μM). Experiments devoid of HRP/P were conducted in parallel. Eugenol (0.2 mM) and glutaraldehyde (5 mM) served as positive and reference controls. Following 24 h incubation, samples were processed and analyzed by HPLC/MS/MS. The peak area of cysteine model peptide was related to the internal standard. Peptide reactivity is given as percent peptide depletion, calculated by comparing the peak area of samples containing ascaridole to corresponding controls.

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Quantification of Cytokines in Cell Culture Supernatants of Ascaridole-Treated MoDC

Treatment of MoDC with 60 μM ascaridole induces maturation. Upregulation of costimulatory molecules, migration and maturation of antigen-presenting cells induced by chemicals are supported by the release of specific cytokines. Therefore, levels of IL-1β, TNF-α, IL-6, and IL-8 were analyzed in cell culture supernatants of ascaridole-treated MoDC (60 μM, 24 h) from 4 (IL-8) to 6 (IL-1β, TNF-α, IL-6) different donors. As expected, levels varied in a donor-dependent manner (Fig. 3). IL-1β was enhanced in supernatants of 3 of 6 donors, TNF-α by all 6 donors, IL-6 by 2 of 6 donors, and IL-8 by all 4 donors investigated. In detail, IL-1β levels in supernatants of individual donors was increased by ascaridole stimulation up to 255%, TNF-α up to 393%, IL-6 up to 263%, and IL-8 up to 201%. Statistical significance was reached for TNF-α and IL-8.

Protein Reactivity Potential of Ascaridole

To gain further experimental support for the skin sensitizing potential of ascaridole, we investigated the protein reactivity potential of ascaridole toward 2 synthetic peptides based on cysteine or lysine. To characterize the reactivity profile of ascaridole, concentrations of peptides and test chemical were chosen based on assay conditions that have previously been optimized toward the prediction of sensitizing potential (Gerberick et al., 2004). We found a clear, albeit limited, potential of ascaridole to interact with the cysteine-containing peptide, resulting in 29% of cysteine depletion after 24 h (Table 1). In contrast, no depletion of the lysine-containing peptide was observed, even when using a 5 times higher concentration of ascaridole. The positive/reference controls p-benzoquinone and DNCB interacted with cysteine and lysine peptides as expected.

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FIG. 2. Early and late maturation of ascaridole-treated MoDC. MoDC were treated with 60 μM ascaridole for 24 and 96 h. Expression of CD86 (A), CD40 (B), CD80 (C), and CD54 (D) on viable cells (PI exclusion) were determined via flow cytometry. Results for ascaridole are shown in light gray, medium in white, and LPS (0.1 μg/ml) in dark gray. Data from 5 to 9 different donors are represented as boxplots with the median MFI (line), 25th to 75th percentiles (box) and minimum to maximum (whiskers). Symbols indicate significant differences (*p < 0.05, paired t test on log-transformed data).
Ascaridole and activated ascaridole, respectively, for 24 h (Fig. 6). Thus, iron-activated ascaridole and its possibly formed products dramatically increase its potential to act as radical inducer. Activation of ascaridole with FeSO₄ for 1 h prior to addition of the cysteine-containing peptide led to a fast and explicit depletion (up to 87%) of the peptide detected after 3, 6, 12, and 24 h (Fig. 4A). In contrast to that, no depletion of the lysine peptide could be detected after incubation with 25 mM activated ascaridole (Fig. 4B), underlining the specific reactivity of ascaridole and possibly formed products of ascaridole toward the cysteine-containing peptide.

We additionally studied the potential impact of enzymatic conversion of the olefin in ascaridole. We used an enzymatic oxidation system comprising a peroxidase since they are known to be expressed in DC (de Oliveira et al., 2014) and likely other skin cells, and whose suitability for activation of a range of chemicals has been demonstrated (Gerberick et al., 2009). This experiment was specifically conducted with deferoxamine as iron chelator to avoid radical side reactions independent of enzymatic reactions (Hoe et al., 1982).

Our results regarding cysteine reactivity of ascaridole in the presence and absence of the HRP/hydrogen peroxide system (HRP/P) under established experimental conditions (Gerberick et al., 2009) did not indicate an increased reactivity after 24 h (Table 2). The observed somewhat reduced depletion in the presence of the enzyme is unclear but was not based on inhibition of the enzymatic activity by ascaridole itself (data not shown). Besides, the positive control eugenol and reference control glutaraldehyde led to results comparable to published data (Troutman et al., 2011). Therefore, we conclude that the olefin in ascaridole might not be oxidized. Similar observations have only been revealed for aliphatic alcohols (Gerberick et al., 2009). Thus, activation of ascaridole depends rather on radical formation conditions resulting in the transformation of ascaridole into more protein-reactive products.

**Induction of HMOX1 Gene Expression in Surrogate DC by Ascaridole**

Activation and mRNA transcription of genes with antioxidant/ electrophile response elements is an event commonly induced by skin sensitizers (OECD, 2012). Mechanistically, this relies on the cytosolic protein Keap1, whose cysteine modifications caused by oxidative or electrophilic stress activates the transcription factor Nrf2. To investigate whether the enhanced potential of activated ascaridole to interact with cysteine-containing peptides also impacts on biological events occurring during the skin sensitization process, we analyzed the expression of the Nrf2 target gene HMOX1. To facilitate sensitive detection of effects, further mechanistic experiments were conducted in THP-1 cells, an established model for DC. HMOX1 gene expression increased and mRNA levels reached significance at higher concentrations of ascaridole (2.7–4.5 mM, 6 h). As expected, activated ascaridole increased mRNA levels significantly at lower concentrations (0.3–0.9 mM) (Fig. 5). Thus, iron-activated ascaridole and its possibly formed products dramatically increase its potential to activate HMOX1 gene expression in THP-1 cells.

**Activation of Surrogate DC Following Incubation With Iron-Activated Ascaridole**

To see whether activation of ascaridole also enhances its potential for DC activation, we measured the upregulation of CD86 and CD54 on THP-1 cells following exposure to 600 and 900 μM ascaridole and activated ascaridole, respectively, for 24 h (Fig. 6). Comparing the results obtained for ascaridole to the THP-1 response to activated ascaridole revealed a significant
upregulation of CD86 (Fig. 6A) and a trend \((p = .08)\) to a significant upregulation of CD54 (Fig. 6B). Ascaridole and activated ascaridole (600 and 900 \(\mu M\)) diminished cell viability to up to 70% (PI exclusion). The positive control DNCB (10 \(\mu M\)) produced results comparable to historical data. FeSO\(_4\) barely modulated expression of CD86 and CD54 at any concentration tested, as shown for the highest concentration applied (180 \(\mu M\)). Thus, radical transformed ascaridole products possess a clearly enhanced potential to activate THP-1 cells.

**DISCUSSION**

The increasing trend of using botanicals and essential oils in consumer products has been suspected as reason for the high frequency of fragrance-related ACD (Johansen and Lepoittevin, 2011; Jack et al., 2013). TTO contains many distinct molecules, thus its sensitizing properties are likely attributable to several components, with the endoperoxide ascaridole being among the suspected molecules. We recently reevaluated its potential under diagnostic patch test conditions (Christoffers et al., 2013, 2014), and found associations of positive elicitation responses with usage of TTO-containing consumer products in some subjects but we also observed positive reactions to ascaridole that were independent of such applications, indicating unknown sources of ascaridole. Furthermore, we observed that the positive reactions were paralleled by increasing cases of irritant reactions. The properties of skin irritants and the potential to induce skin sensitization are not mutually exclusive, as many sensitizers also provide an inherent irritancy. Thus, these results made demands for further experiments to substantiate the human sensitization potential of ascaridole.

Based on investigations demonstrating that chemical allergens but not irritants (Aiba et al., 1997) induce upregulation of costimulatory molecules (B-7 family CD80/CD86; Linsley et al., 1994) and adhesion molecules, which account for a successful T cell recognition of an antigen and subsequent proliferation, we explored whether ascaridole is able to activate and mature human MoDC. Indeed, ascaridole increased cell surface expression of CD86, CD80, and CD40. CD40 is, together with its ligand (CD40L), an important receptor that orchestrates differentiation of antigen-specific CD8\(^+\) T-cell responses (Hernandez et al., 2008). We additionally observed an upregulation of CD54. This molecule—together with its binding partner LFA-1—has several important functions within the formation of the immunological
synapse: facilitation of T-cell activation by promoting efficient adhesion of T cells to antigen-presentation cells and the transmission of intracellular signals that synergize with T-cell receptor-mediated signals to promote T-cell proliferation and differentiation (Grakoui et al., 1999).

DC maturation and immunity is critically dependent on cytokines released by DC among others. Fully mature DC induce immunity, whereas semimature DC fail, although they are phenotypically mature but are characterized by a defect to release inflammatory cytokines (Lutz and Schuler, 2002). Thus, 1 decoy immunogenic signal is the release of proinflammatory cytokines to amplify and direct the type of immune response (Curtisinger et al., 1999). In response to ascaridole, we found significantly enhanced cytokine release of TNF-α and IL-8 but also enhanced levels of IL-6, IL-1β but no IL-10 (data not shown). Especially, IL-8 was proposed to play a pivotal role in the induction of skin sensitization by chemicals (Toebak et al., 2006). In sum, the observed DC maturation together with the observed mediator release are supportive for ascaridole being capable of inducing full DC maturation and not only being an irritant but a human sensitizer.

The ability of chemicals to modify proteins is a fundamental event in the induction of sensitization. Supporting our conclusion drawn from DC maturation, we found a clear, albeit limited, reactivity of ascaridole toward cysteine peptides. Similar to many other skin sensitizers including hydroperoxides, ascaridole preferentially interacted with cysteine- but not lysine-containing peptides.

Reactivity of the fairly stable endoperoxide ascaridole may, similar to other peroxides/hydroperoxides, critically depend on the formation of radicals. To support this hypothesis we studied the consequences in the presence of a radical inducer (catalytic amount of iron [Brown et al., 1962]), that initiates the cleavage of the endoperoxide. Because radicals are rather soft electrophiles (Aptula et al., 2009), we postulated to find covalent binding toward sulphydryl group of amino acid side chains. Indeed, compared with ascaridole itself, radical transformed ascaridole resulted in about 3-fold higher reactivity toward the cysteine-containing peptide. Unfortunately, our experiments cannot provide definitive answers to whether radical formation alone and/or intramolecular rearrangements such as epoxidation, or oxidoreduction processes have occurred. The latter aspect needs further research eg, via radical trapping using scavenger agents. Nevertheless, others provided evidence that the cleavage of the endoperoxide bridge generates oxygen- and carbon-centered radical intermediates arising from Fe(II)-induced (FeSO₄ forms Fe(II) aquo complexes in solution) degradation of ascaridole with subsequent formation of the diepoxide isoascaridole (Turner and Herz, 1977a).

In contrast, and as indicated by our data, activation of ascaridole in the presence of the heme-containing HRP, a peroxidase with very broad substrate specificity, is not likely to occur. Thus, and as hypothesized elsewhere (Schmidt, 2007) the iron catalyzed reaction is—beside autoxidation by air exposure and metabolic conversion—an important activation mechanism for chemicals.

Keap1 is a cytosolic protein that senses oxidative and electrophilic stress via oxidation or chemical modification of cysteine residues, which results in the activation of the transcription factor Nrf2 (Nrf2-Keap1-ARE pathway) (Kensler et al., 2007). In mice, a role of Nrf2 in contact hypersensitivity has been demonstrated (El Ali et al., 2013). Nowadays, the activation of Nrf2 is used as the mechanistic basis for several cell-based assays for the identification of skin sensitizing chemicals in keratinocytes (Emter et al., 2010). MoDC and THP-1 cells (Migdal et al., 2013). To further link the very earliest events during which ascaridole forms stable associations with likely cysteine-containing proteins with biological events during acquisition of sensitization, we analyzed the expression of the Nrf2 target gene HMOX1. In line with the observed enhanced reactivity of radical transformed ascaridole toward cysteine peptides, we found that activation of ascaridole enhances its capacity to upregulate the Nrf2 target gene HMOX1 in THP-1 cells.

Finally, we addressed the question whether this enhanced reactivity of radical transformed ascaridole also affects its potential for DC activation by analyzing the upregulation of relevant cell surface molecules on THP-1 cells. Indeed, we observed a significantly augmented CD86 and CD54 surface expression on THP-1 cells after treatment with radical transformed ascaridole compared with ascaridole itself. Because the radical inducer iron is present in skin cells (Keyse and Tyrrell,


