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Toluene diisocyanate exposure and autotaxin–lysophosphatidic acid signalling

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ABSTRACT

Toluene diisocyanate (TDI) is a reactive chemical used in manufacturing plastics. TDI exposure adversely affects workers' health, causing occupational asthma, but individuals differ in susceptibility. We recently suggested a role for signalling mediated by the enzyme autotaxin (ATX) and its product, lysophosphatidic acid (LPA), in TDI toxicity. Here we genotyped 118 TDI-exposed workers for six single-nucleotide polymorphisms (SNPs) in genes encoding proteins implicated in ATX–LPA signalling: purinergic receptor P2X7 (*P2RX7*), C−C motif chemokine ligand 2 (*CCL2*), interleukin 1β (*IL1B*), and caveolin 1 (*CAV1*). Two *P2RX7* SNPs (rs208294 and rs2230911) significantly modified the associations between a biomarker of TDI exposure (urinary 2,4-toluene diamine) and plasma LPA; two *IL1B* SNPs (rs16944 and rs1143634) did not. *CAV1* rs3807989 modified the associations, but the effect was not statistically significant ($p = 0.05–0.09$). *In vitro*, TDI-exposed bronchial epithelial cells (16HBE14o-) rapidly released ATX and IL-1β. P2X7 inhibitors attenuated both responses, but confocal microscopy showed non-overlapping localizations of ATX and IL-1β, and down-regulation of CAV1 inhibited the ATX response but not the IL-1β response. This study indicates that P2X7 is pivotal for TDI-induced ATX–LPA signalling, which was modified by genetic variation in *P2RX7*. Furthermore, our data suggest that the TDI-induced ATX and IL-1β responses occur independently.

1. Introduction

Isocyanates, such as toluene diisocyanate (TDI), are highly reactive compounds used in industrial production of polyurethane plastic. Polyurethane products include rigid and flexible foams and plastics, lacquers, adhesives, surface coatings, and synthetic fibres. Exposure to isocyanates is one of the leading causes of occupational asthma (OA) (Gotzev et al., 2016; Wisnewski et al., 2013) and isocyanates cause symptoms in the eyes, the upper and the lower airways, and eczemas of the skin (Baur et al., 1994; Littorin et al., 2007). Although the isocyanate-related symptoms are similar to those of an allergic reaction, affected individuals rarely have specific antibodies, and if found, the antibodies do not necessarily correlate with symptoms (Liu and Wisnewski, 2003; Swierczynska-Machura et al., 2015; Wisnewski and Jones, 2010). Furthermore, not all workers who are exposed to isocyanates develop symptoms, suggesting that some individuals might be more susceptible to isocyanates than others (Maestrelli et al., 2009). Genome-wide association studies on patients with isocyanate-related OA have found correlations between isocyanate-related OA and genes associated with immune pathways, such as antigen processing and presentation (Yucesoy et al., 2015) as well as cell–cell adhesion (Bernstein et al., 2012). Candidate-gene studies on inflammatory response genes have found modifying effects on isocyanate-related OA and airway symptoms (Broberg et al., 2008; Yucesoy et al., 2016).

Although the genetic studies imply an immune-related component in isocyanate-related OA, the mechanisms behind the toxicity of isocyanates remain mainly unknown. In a previous study, we showed that TDI in nanomolar concentrations induced the enzyme autotaxin (ATX; also known as ectonucleotide pyrophosphatase/phosphodiesterase family member 2, E-NPP 2, encoded by the *ENPP2* gene) in human lung epithelial cell cultures (Broström et al., 2015). We also found that among TDI-exposed workers, blood levels of the main product of ATX, lysophosphatidic acid (LPA), correlated with TDI exposure and mild respiratory symptoms. These data indicated that low-level exposure to TDI influences the ATX–LPA pathway and suggested a role of ATX–LPA signalling in TDI toxicity. Furthermore, in cell cultures, we found a significantly lower ATX response to TDI after reducing the expression of purinergic receptor P2X, ligand-gated ion channel, 7 and 4 (P2X7 and

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P2X4), indicating a key role for these proteins in the TDI–ATX interaction.

In an effort to further understand TDI toxicity, we identified SNPs in selected genes of possible importance for ATX–LPA signalling, including *P2X7* (official gene name *P2RX7*), *IL-1β* (*IL1B*), *CCL2*, and *CAV1*. P2X7 is a member of the ATP-gated, transmembrane, ion channel-forming family. The channels form as multimers of several subunits (North, 2002). *P2RX7* is expressed in several tissues in the human body, including lung epithelium (Collo et al., 1997; Uhlén et al., 2015; www.proteinatlas.org). P2X4 is translocated to the plasma membrane by CCL2 (C−C motif chemokine ligand 2) (Toyomitsu et al., 2012), which has also been suggested as a biomarker for isocyanate OA (Bernstein et al., 2002), and in our previous work we found that pretreating cells with CCL2 before exposing them to TDI increased the ATX response (Broström et al., 2015). Activated P2X7 receptors are implicated in the release of the pro-inflammatory cytokine interleukin 1β (IL-1β). Like extracellular ATP, IL-1β is considered a danger signal for cells (Trautmann, 2009), and IL-1β spreads inflammation signalling (Ferrari et al., 2006). IL-1β can overcome the negative feedback LPA may exert on ATX (Benesch et al., 2015), thereby increasing the amount of LPA produced. IL-1β has been suggested to mediate TDI-related airway hyperresponsiveness and inflammation in mice (Johnson et al., 2005). P2X7 is present in caveolae in airway epithelial cells, lipid-rich invaginations in the cellular membrane that contains caveolin 1 (CAV1), and down-regulation of CAV1 caused a reduction in P2X7 expression (Barth et al., 2007).

The aim of this study is to clarify the role of the P2X7–ATX–LPA signalling pathway in TDI toxicity. Therefore, we analysed whether SNPs in *P2RX7*, *IL1B*, *CCL2*, and *CAV1* modified LPA levels and isoforms in our cohort of TDI-exposed humans. We performed additional cell studies in a human non-cancer bronchial epithelial cell model. Our results show the central role for P2X7 and indicate that responses involving ATX and IL-1β can be separated.

2. Materials and methods

2.1. Study group

Blood and urine samples were collected from 118 workers from nine polyurethane plants (Table 1). The workers were primarily exposed to the isocyanate TDI, having an occupational airborne TDI exposure below 5 ppb, the Swedish occupational exposure limit for an 8-h time-weighted average, at the time of sample collection (Sennbro et al., 2004a). Further, all participants were interviewed for occupational and medical history, including smoking habits, and the presence of eye and airway symptoms such as wheezing and sneezing over the last year (Littorin et al., 2007). Informed consent was obtained from all participants, and ethical approval was acquired from the ethical review board in Lund, Sweden.

2.2. Analysis of biomarkers in urine and plasma

Blood and urine samples were collected and analysed by LC/MS/MS for the 2,4-TDI exposure biomarker (2,4-toluene diamine (2,4-TDA)) in hydrolysed urine as described (Sennbro et al., 2003; Sennbro et al., 2004b). The four most common species of LPA, i.e. 16:0, 18:0, 18:1 and 20:4 in plasma were analysed by LC/MS/MS with a preceding methanol extraction as described (Broström et al., 2015).

2.3. Selection of SNPs

SNPs in *P2RX7*, *IL1B*, *CCL2*, and *CAV1* were identified in NCBI dbSNP (https://www.ncbi.nlm.nih.gov/projects/SNP) and sorted by their minor allele frequency (MAF) in the HapMap data for CEU (Utah residents with Northern and Western European ancestry). SNPs with a MAF > 5% that were reported to have a functional effect on inflammation and/or airway disease were considered for the analysis. The two SNPs in *P2RX7* were not in linkage disequilibrium, and neither were the two SNPs in *IL1B* (https://www.ensembl.org/). Because *P2X4* had comparably few identified SNPs, only 3 with a MAF > 5%, and none of them with a known functional effect, this gene was not included in the final selection. Table 2 lists the SNPs included in the study.

Table 1
Characteristics of the study group of toluene diisocyanate (TDI)-exposed workers.

Variables	Characteristics
Age, mean/median (range)	37/36 (18–62)
Female/ total	23/118 (20%)
Smokers/ total[a]	70/116 (60%)
Sneezing/ total[b]	50/115 (44%)
Wheezing/ total[b]	34/115 (30%)
Gene, SNP and genotype	n (%)
CAV1 rs3807989 GG/GA/AA	41/56/20 (35/48/17)
IL1B rs16944 GG/GA/AA	52/55/10 (44/47/9)
IL1B rs1143634 GG/GA/AA	59/52/6 (50/44/5)
P2RX7 rs208294 CC/CT/TT	28/68/21 (24/58/18)
P2RX7 rs2230911 CC/CG/GG	97/17/3 (83/14/3)
CCL2 rs1024611 AA/AG/GG	59/50/8 (50/43/7)
Analytes	Mean/median (range) nmol/L
Urinary 2,4-TDA[c]	43/23 (0.4–623)
Plasma LPA[c] 16:0	854/681 (133–2942)
Plasma LPA 18:0	160/136 (40–477)
Plasma LPA 18:1	477/422 (85–1412)
Plasma LPA 20:4	713/621 (206–2236)
Total plasma LPA	2204/1917 (491–6445)

[a] Smokers were defined by the question: “Have you, during the last year, been smoking? Smokers include both regular and seldom/party smokers.

[b] Sneezing and wheezing were defined by the question: “Have you experienced any (wheezing/sneezing) during the last year?”

[c] Abbreviations: TDA = 2,4-toluene diamine, the exposure biomarker for TDI; LPA = lysophosphatidic acid.

2.4. Genotyping

DNA had previously been extracted from white blood cells by QIAamp DNA Blood Mini extraction kit (Broberg et al., 2008). Allelic discrimination, using TaqMan SNP genotyping assays (Life Technologies, Carlsbad, California), was performed on an ABI 7900HT (Applied Biosystems, Foster City, CA, USA) according to the manufacturers' instructions. For quality control, 5% of the samples were rerun, and all samples were in concordance with the original analysis.

2.5. Cell culture

Human bronchial epithelial cells (16HBE14o-), transformed with SW40 large T antigen, were provided by Prof. Dieter C. Gruenert (University of California, San Francisco, CA; Cozens). Cells were cultured in EMEM (Lonza, Basel, Switzerland) medium supplemented with 10% inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. Cells were maintained at 37 °C in humidified air containing 5% CO_2. TDI was purchased from Sigma-Aldrich (St. Louis, MO). TDI stock (10 or 1 μM) was prepared in DMSO, and the final DMSO concentration in the medium was ≤0.1%. All experiments were repeated at least three times with different cell batches.

2.6. siRNA Transfection

P2X7 small interfering RNA (siRNA), and control siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Cells were seeded in six-well plates with 40–50% confluence and transfected with indicated siRNA for 40 h using Lipofectamine RNAiMAX in accordance with the protocol from the manufacturer (Invitrogen, Carlsbad, CA).