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Toluene diisocyanate: Induction of the autotaxin-lysophosphatidic acid axis and its association with airways symptoms

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ABSTRACT

Diisocyanates are industrial chemicals which have a wide range of applications in developed and developing countries. They are notorious lung toxicants and respiratory sensitizers. However, the mechanisms behind their adverse effects are not adequately characterized. Autotaxin (ATX) is an enzyme producing lysophosphatidic acid (LPA), and the ATX-LPA axis has been implicated in lung related inflammatory conditions and diseases, including allergic asthma, but not to toxicity of environmental low-molecular-weight chemicals.

We investigated effects of toluene diisocyanate (TDI) on ATX induction in human lung epithelial cell models, and we correlated LPA-levels in plasma to biomarkers of TDI exposure in urine collected from workers exposed to <5 ppb (parts per billion). Information on workers' symptoms was collected through interviews.

One nanomolar TDI robustly induced ATX release within 10 min in vitro. A P2X7- and P2X4-dependent microvesicle formation was implicated in a rapid ATX release and a subsequent protein synthesis. Co-localization between purinergic receptors and ATX was documented by immunofluorescence and confocal microscopy. The release was modulated by monocyte chemoattractant protein-1 (MCP-1) and by extracellular ATP. In workers, we found a dose–response relationship between TDI exposure biomarkers in urine and LPA levels in plasma. Among symptomatic workers reporting "sneezing", the LPA levels were higher than among non-symptomatic workers.

This is the first report indicating induction of the ATX-LPA axis by an environmental low-molecular-weight chemical, and our data suggest a role for the ATX-LPA axis in TDI toxicity.

Introduction

Toluene diisocyanate (TDI) and other diisocyanates are chemicals widely used in polyurethane production with applications ranging from adhesives and lacquers to rigid and flexible foams. Despite strict regulations and low occupational exposure limits (OELs) in industrialized countries, diisocyanates were estimated to remain among the European top ten occupational hazard research priorities (Brun, 2008), and are the agents most frequently causing occupational asthma (Bakerly et al., 2008). Other symptoms resemble a common cold or allergy and include irritation of the eyes, throat and upper and lower airways (Baur et al., 1994). Isocyanates may act both as damaging irritants and as sensitizers. While the OELs are set to prevent acute reactions and sensitization, already sensitized individuals may react to levels well below the OELs. As recently summarized in (Verschoor and Verschoor, 2014), sensitization may occur from acute high concentration exposures or from continuous low level concentration exposures, through exposure to the skin and/or airways and there seem to be differences in individual susceptibility as well. Most often, both increased total IgE and isocyanate specific IgE are lacking in exposed individuals, but when present they may coincide with diisocyanate asthma (Liu and Wisnewski, 2003). The lack of knowledge regarding the underlying mechanisms restricts development of diagnostics and prevention.

Autotaxin (ATX) is a secreted lysophospholipase which produces lysophosphatidic acids (LPAs) from lysophosphatidylcholine. ATX inhibition drastically reduces LPA plasma levels, indicating a rapid turnover and a dominant role for ATX in LPA production (Albers et al., 2010). LPAs are lipid signaling molecules that bind to transmembrane G protein-coupled receptors and LPA might be released from ATX at the cognate receptor site in a highly localized fashion (Tabchy et al., 2011). LPA receptors mediate basic cellular functions including proliferation, apoptosis and migration (Moolenaar and Perrakis, 2011).

Abbreviations: ATX, autotaxin; LPA, lysophosphatidic acid; TDI, toluene diisocyanate; oATP, oxidized ATP; TDA, toluene diamine; MCP-1, monocyte chemoattractant protein-1.

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ATX induction has been associated with cancer progression (Tabchy et al., 2011; Kadekar et al., 2012), rheumatoid arthritis (Nikitopoulou et al., 2012), idiopathic pulmonary fibrosis (Oikonomou et al., 2012) and also with lung fibrosis induced by the cancer drug bleomycine (Budd and Qian, 2013). Recently a study indicated a role for ATX in allergic airway inflammation after allergen challenge (Park et al., 2013) and remaining questions include cellular mechanisms behind ATX induction (Georas, 2013). This literature suggests a perhaps specific role of ATX in lung remodeling and pathology, but as far as we know, ATX induction has never been associated with lung toxicity induced by low-molecular-weight environmental chemicals.

In this study we investigate a possible role of the ATX-LPA axis in toluene diisocyanate (TDI) toxicity. We report that low concentrations of TDI rapidly release ATX from lung epithelial cells via a purinergic receptor-dependent mechanism. We also show correlations between TDI exposure biomarkers and plasma LPA levels in exposed workers and report correlations between TDI exposure, airway symptoms and LPA levels in plasma.

Methods

Cell culture. Non-small cell lung cancer cells, A549, were purchased from ATCC (American Type Culture Collection, Manassas, VA). Cells were grown in Dulbecco's modified Eagle's medium (DMEM), with 10% inactivated calf serum, penicillin/streptomycin, and 1 mM sodium pyruvate. Serum starved cells were cultured with medium supplemented with 0.1% serum for 24 h. Immortal human bronchial epithelial cells were (16HBE14o−) incubated as described in (Cozens et al., 1994). In brief, pre-coated conditions were used. Coating was done with bovine serum albumin (BSA, Sigma Aldrich) (0.01 mg/ml), collagen I from bovine 0.03 mg/ml (Corning 354231), human fibronectin 0.01 mg/ml (Corning 354008). Cells were grown in Minimum Essential Medium Eagle (EMEM) with L-glutamine (Lonza, BE12-611F), the medium was supplemented with 10% inactivated fetal bovine serum (FBS), and penicillin–streptomycin. Cell viability in cultures was routinely checked.

Reagents. 2,4-and 2,6-toluene diisocyanate (TDI), adenosine 5′-triphosphate periodate oxidized sodium salt (ATP), 2′(3′)-O-(4-benzoyl–benzoyl) adenosine 5′-triphosphate triethylammonium salt (o-ATP), mixed isomer of 2′(3′)-*O*-(4-Benzoylbenzoyl)adenosine-5′-triphosphate tri(triethylammonium) salt (BzATP), N-acetyl cysteine (NAC), Brilliant Blue G (BBG), MCP-1 (CCL2) and KN-62 were purchased from Sigma-Aldrich (St. Louis, MO). ATX, Cdk2 (sc-163), P2X4 and P2X7 antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and P2X4 (for confocal microscopy and proximity ligation assay) from Abnova (Taipei, Taiwan). The specificity of the P2X7 antibodies was checked by overexpressing experiments. Dulbecco's modified Eagle's medium (DMEM), Fetal Bovine Serum (FBS), penicillin–streptomycin and sodium pyruvate were from Invitrogen (Carlsbad, CA, USA), secondary antibody conjugated with FITC (fluorescein isothiocyanate) from Dako (Glostrup, Denmark). TDI stock was prepared in DMSO and final DMSO concentration in the medium was less than 0.2%. In experiments with multiple TDI concentrations, dilutions were used so a single final DMSO concentration was obtained. Data are presented as comparisons to the DMSO control.

siRNA transfection. Cells were transfected with P2X4 and P2X7 small interfering RNA (siRNA) or control siRNA (Invitrogen, Paisley, UK) for 72 h by using Lipofectamine RNAiMAX (Invitrogen, Paisley, UK) according to the manufacturer's instructions.

Fluorescence and confocal microscopy. In fluorescence microscopy (immunocytochemical staining), cells were fixed in 4% formaldehyde for 20 min. After fixation the cells were stained with antibodies for P2X7, ATX or P2X4. After incubation with primary antibodies at 4 °C overnight, secondary antibody conjugated with FITC were applied for 1 h. No staining was detected when the primary antibodies were omitted. For confocal microscopy cells were fixed with 4% formaldehyde, permeabilized with 0.2% Triton X-100 in 2% BSA buffer. Immunostaining was performed using rabbit polyclonal ATX antibody and mouse monoclonal P2X4 antibodies, or polyclonal P2X7 antibodies. Secondary rabbit antibodies conjugated with Alexa 488 were employed. Samples were mounted in DAPI. Fixed cells were analyzed with a Zeiss LSM 510 META confocal laser scanning microscope (Zeiss, Oberkochen, Germany) equipped with a ×63 Plan-Aoil-immersion lens. An argon laser was excited at 488 nm and fluorescence image was recorded from 500 nm to 550 nm.

Proximity ligation assay. Proximity ligation assay (PLA) was performed according to the manufacturer's protocol using the Duolink detection kit with PLA PLUS and MINUS probes for mouse and rabbit (Olink Bioscience, Uppsala, Sweden).

Western blotting. Cells were washed with PBS and lysed in IPB-7 (1 mg/mL phenylmethylsulfonyl fluoride, 0.1 mg/mL trypsin inhibitor, 1 mg/mL aprotinin, 1 mg/mL leupeptin, 1 mg/mL pepstatin, 1 mmol/L Na3VO4, and 1 mmol/L NaF). The samples were subjected to SDS-PAGE and thereafter blotted onto a PVDF membrane (Bio-Rad, Hercules, CA). The protein bands were probed using antibodies as indicated in figures. Proteins were visualized with the ECL procedure (Amersham Biosciences, GE Healthcare, Little Chalfont, UK). The Western blotting results were analyzed with NIH Image 1.62 software. All experiments were repeated at least three times with different batches of cells. The extracellular protein precipitation was performed by using acetone according to the protocol for acetone precipitation of protein (Pierce Biotechnology, Thermo Fisher Scientific, Waltham, MA).

Clinical samples. The clinical samples consisted of 118 individuals employed at 9 small isocyanate companies handling mainly TDI in southern Sweden (Table 1). The air levels in these factories were below the Swedish OEL, which was 5 ppb (8 h time-weighted average) at the time of sample collection. Also, 86 referent workers from 4 different work places in the same area with no known occupational exposure to isocyanates were examined. Plasma and urine samples had been collected and analyzed for exposure biomarkers, which were presented in previous studies (Sennbro et al., 2004a, 2004b, 2005). In the present study, we have used the 2,4- and 2,6-toluene diamine (TDA) exposure biomarkers in (hydrolyzed) urine. The samples included here are those for whom urine TDA levels were available and plasma samples could be retrieved and analyzed for LPA. The samples had been collected after at least 4 h of work on days not following weekends or holidays (Sennbro et al., 2004a, 2004b).

All participants were interviewed at two time points about the following symptoms: wheezing, hacking cough, hoarseness, runny nose, stuffy nose, sneezing, nosebleeds and symptoms from the eyes. Once a physician phrased the questions “Have you had attacks of … during the last year?” (Littorin et al., 2007) and once a nurse when collecting the blood samples asked “Have you experienced … during the last three days?” Three subjects, who did not answer at all or had given unclear answers, were excluded from the analysis of interactions presented in Table S2.

Informed consent was obtained from all participants, and ethical approval was acquired from the ethical review board in Lund, Sweden.

Plasma LPA analysis. The 204 plasma samples were analyzed by liquid chromatography with tandem mass spectrometry (LC/MS/MS) for detection of LPA with a preceding methanol extraction method described by Zhao and Xu (Zhao and Xu, 2010) with slight modifications. Briefly, plasma samples were added with methanol and 17:0 LPA as an internal