

Original Article

Toluene diisocyanate (TDI) induces calcium elevation and interleukine-4 (IL-4) release – Early responses upon TDI stimulation

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ABSTRACT — Occupational exposure to toluene diisocyanates (TDI) may cause asthma. In asthma patients, the allergic syndromes correlate cytokine production with the elevation in cytosolic calcium concentration $[Ca^{2+}]_c$ of lymphocytes in airway. We previously found TDI induces calcium signaling in neuronal cells. TDI mainly gets into human body via inhalation; therefore this study investigated the possibility of TDI inducing the changes in $[Ca^{2+}]_c$ in airway. We used human lung epithelial cell line H1355, human T-cell line Jurkat, and human neuroblastoma SH-SY5Y cells to present the kinds of cells existing in airway. The changes of $[Ca^{2+}]_c$ were measured by Fura-2 fluorescent dye. Results show that TDI induced an elevation in $[Ca^{2+}]_c$ in those cell lines and two primary isolated cells, bovine adrenal chromaffin cells and human white blood cells. Cytokine release and their gene expressions of Jurkat cells and human white blood cells were measured by ELISA and reverse transcription polymerase chain reaction. TDI acutely promoted the interleukine-4 (IL-4) release significantly in both Jurkat cells and human white blood cells. TDI-induced IL-4 release was suppressed in the presence of 1,2-bis- (O-aminophenoxy)ethane-N,N,N',N'- tetraacetic acid (BAPTA), an intracellular Ca^{2+} chelator, in Jurkat cells. In the hand of gene expression, TDI induced an increase in the mRNA level of TNF- α and IL-4 in Jurkat cells. We conclude that the release of IL-4 were coupled with the elevation in $[Ca^{2+}]_c$ induced by TDI. Further studies are required to clarify the roles of TDI-induced IL-4 secretion in acute inflammation.

Key words: TDI, Calcium signaling, Cytokine release, Jurkat cell, White blood cells

INTRODUCTION

Exposure to diisocyanates is a well known occupational hazard causing asthma and allergic rhinitis (Lemière *et al.*, 1996). Occupational exposure to 2, 4-Toluene diisocyanate (TDI) can occur among workers involved in its manufacture, handling and use (Tarlo *et al.*, 1997). The general public may be exposed to TDI through emissions from urethane foam production facilities. In the induction of isocyanate asthma, inflammatory and neurogenic factors might be involved (Mapp *et al.*, 1994b;

Scheerens *et al.*, 1996). Vanoirbeek found in 2009 that an elevation in immunoglobulin-E (IgE) under TDI treatments (Vanoirbeek *et al.*, 2009) which can explain the TDI-induced allergy although TDI can induce allergies through IgE independent pathways mentioned in some literature. The IgE release from B cells occurs in the presence of interleukine-4 (IL-4) which can be released from T helper cell type 2 (Th2 cells). TDI is well known to stimulate Th2 cells to elevate IL-4 levels (Ban *et al.*, 2006; Johnson *et al.*, 2007; Sun *et al.*, 2007). Not only chronic asthma but also acute reactions induced by TDI

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are shown in the literatures (Mapp *et al.*, 1988; McKay and Brooks, 1984; Raulf-Heimsoth and Baur, 1998). The expression of IL-4 protein has been found to be increased only in the asthmatic subjects tested after 48 hr exposure to TDI (Maestrelli *et al.*, 1997). In murine, mRNA of IL-4, TNF- α , etc. expressed in higher levels after TDI challenge (Matheson *et al.*, 2001). Short term exposure of TDI vapor also resulted in the production of IL-4 (Kouadio *et al.*, 2005). Cytokine production by Th2 Cells increased both in the bronchial mucosa and peripheral mononuclear cells of patients with TDI-induced asthma (Maestrelli *et al.*, 1997; Lee *et al.*, 1998; Lummus *et al.*, 1998). IL-6-like substances and IL-6 gene expression were also reported from bronchial epithelial cells *in vitro* exposed to isocyanates (Mattoli *et al.*, 1991). However, the mechanisms of TDI-induced acute responses are still unclear.

Calcium coupled neurotransmitter secretion responses have been well-known (Douglas and Rubin, 1961; Douglas and Poisner, 1964). Calcium-coupling secretion responses are also known in IgE release (Pecht and Corcia, 1987; Liu *et al.*, 1987). In bronchial asthma patients, there are increased intracellular free calcium concentration ($[Ca^{2+}]_i$) in their leucocytes and platelets (Chhabra *et al.*, 1999; Efendieva *et al.*, 1988; Kuroda *et al.*, 1995). We previously found that TDI induces an increase in $[Ca^{2+}]_i$ in human neuroblastoma SH-SY5Y cells (Chiung *et al.*, 2005; Liu *et al.*, 2006a, 2006b). However, how those acute cellular responses bring out series of follow-up complicate mechanisms of TDI-induced asthma is not yet known.

Inhalation is the main route to let TDI get into human body. Through airway to circulation, some tissue cells exist, such as epithelial cells, lung cells, white blood cells, nerve cells and so on. The character of TDI inducing an elevation in $[Ca^{2+}]_i$ in these cells is not well known. Its effect on cytokine production is also not reported. In the present study, the effects of TDI on the change of $[Ca^{2+}]_i$ in human lung epithelial cell line (H1355), human epithelial HeLa cells, human T-cell line Jurkat, and human neuroblastoma SH-SY5Y cells were investigated. Two primary isolated cells were used also, named bovine adrenal medullary chromaffin cells representing the normal neuron, and human white blood cells representing the immune effect cells. TDI was evidenced to induce an elevation in $[Ca^{2+}]_i$ of those cells in this study. The following cytokine releases were found subsequently in human T-cell line Jurkat cells and human white blood cells. This study is going to establish the linkage between cytokines release and calcium signaling induced by TDI.

MATERIALS AND METHODS

Materials

Human lung epithelial cell line (H1355) (CRL-5865), human epithelial HeLa cells, human T-cell line Jurkat and human neuroblastoma SH-SY5Y cells were obtained from American Type Culture Collection. TDI (2,4-toluene diisocyanate), 1,2-bis-(O-amino- phenoxy) ethane-N,N,N',N'- tetraacetic acid (BAPTA) and ethylene glycol tetra-acetic acid (EGTA) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Fura-2/AM was purchased from Molecular Probe (Eugene, OR, USA). Dulbecco's modified Eagle's medium and penicillin-streptomycin were purchased from Gibco BRL (Gaithersburg, MD, USA).

Cell culture

The human epithelial cell line, HeLa, was cultured in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum in a 5% CO₂ humidified incubator at 37°C (James and Giorgio, 2000). The human lung epithelial H1355 cells and The human T leukemia Jurkat cells were cultured in a RPMI1640 medium supplemented with 10% fetal bovine serum (Cheadle *et al.*, 2005; Lin *et al.*, 2004). The human neuroblastoma SH-SY5Y cells were cultured in MEM medium supplemented with 10% fetal bovine serum (Liu *et al.*, 2006a).

Primary cell isolation

Bovine adrenal medullary cells were isolated according to a previously described method (Liu *et al.*, 2006b). Briefly, adrenal glands were perfused first with perfusion buffer, and then with a collagenase solution. After separation from the cortex, the medulla was cut into small pieces and digested with the same collagenase solution at 37°C. Fresh isolated cells were used for experiments.

Human white blood cells (WBC) were isolated from the peripheral blood. Blood sample was obtained from three healthy male donors; all of them non-smokers and had not been exposed to TDI. Venous blood (10 ml) was collected under sterile conditions in Heparinized Vacutainer Tubes (Becton Dickinson, NJ, USA) containing lithium heparin as anticoagulant. The whole blood was treated with lysis buffer twice for 10 sec to get rid of erythrocytes.

Plasma samples of study subjects

The specified TDI allergic subjects were diagnosed according to the results in questionnaires about symptoms, examinations of skin tests and anti TDI antibodies. Because all the subjects worked in the factories using TDI,

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and the blood samples were obtained during their working hours. All of them gave informed consent. According to the diagnosis as above, workers were divided into TDI allergic, non-TDI allergic control and non-allergic control groups. No subject was using allergic drugs.

[Ca²⁺]_i measurements

The cells were loaded with fura-2 by incubation (5×10^6 cells/ml) using 10 μ M fura-2 AM at 37°C for 30 min. Cells were then washed twice with a loading buffer (150 mM NaCl, 5 mM KCl, 2.2 mM CaCl₂, 1 mM MgCl₂, 5 mM glucose, 10 mM HEPES, pH 7.4). The vehicle, TDI (freshly prepared in DMSO) was individually added to the fura-2 loaded cells and the fluorescence was measured at 340 nm, 380 nm excitation and 505 nm emission. Cytosolic Ca²⁺ concentration ([Ca²⁺]_i) was calculated using a fluorescence ratio of 340 nm to 380 nm (Grynkiewicz *et al.*, 1985). R_{max} was achieved by adding 0.01% digitonin to a cuvette at the end of each experiment; Excess EGTA was subsequently added to obtain R_{min}. A K_d of 224 nM Ca²⁺ for fura-2 was used.

Measurements of cytokine release

Cytokine releases induced by TDI were measured following steps as stimulation, neutralization and collection. Supernatants of the TDI-treated WBC or Jurkat cells were collected after incubation (5×10^7 cells/ml) with TDI with DMSO as a vehicle. The experiment initiated as adding the stimulation of freshly prepared TDI. Ammonium bicarbonate was added after 5 min of TDI activation, the supernatant was collected from this time point. The supernatants were collected at 5 min, 5-25 min or 0-25 min. Assays for IFN- γ , TNF- α , IL-4 and IL-6 were performed by using human cytokine antibodies (Peprotech Inc., NJ, USA) and commercially available reagents for ELISA (Bionova Inc., NJ, USA).

The cytokine amounts in the plasma sample collected from workers were measured as above. Concentrations were determined from the triplicate results (one person) which were calculated from the standard curves performance by recombinant cytokines. The cytokine levels were detected by using human cytokine antibodies (Peprotech Inc., NJ, USA) and commercially available reagents for ELISA (Bionova Inc., NJ, USA), and using isotype as control.

Quantification of cellular mRNA encoding cytokines

After treatments with TDI, Jurkat cells were neutralized, washed and resuspended in 10% FBS-RPMI and incubated for 3 hr. The expression of cytokine transcripts

was analyzed by reverse transcriptase–polymerase chain reaction (RT-PCR) of total RNA extracted with the Trizol reagent (Ambion Inc., TX, USA), and remove genomic DNA with DNase (Ambion Inc.). The reverse transcription was performed using a Superscript III kit with random hexamer primers and PCR products were amplified with Platinum Taq polymerase (Invitrogen) for 18 to 35 cycles. Specific primers were as follows: TNF- α : 5'-AGCCCATGTTGTAGCAAA-CC-3' and 5'-GGAAGACCCCTCCCAGATAG-3', IL-4: 5'-ACTGCTTCCCCCTCTGTTCT-3' and 5'-CTCTGGTTG-GCTTCCTTCAC-3', IL-6: 5'-ATGTAGCCGCCCCACACAGA-3' and 5'-CATCCATCTTTTCAGCCAT-3' and GAPDH: 5'-ACCACAGTCCATGCCATCAC-3' and 5'-TCCACCACCCTGTTGCTGTA-3'. The annealing temperature used in this study was 55°C for TNF- α , IL-6 and GAPDH, 58°C for IL-4. The PCR products were subjected to agarose gel electrophoresis (2%), stained with ethidium bromide, photographed and then analyzed with the bands using the FluorChem8990 program. The rela-

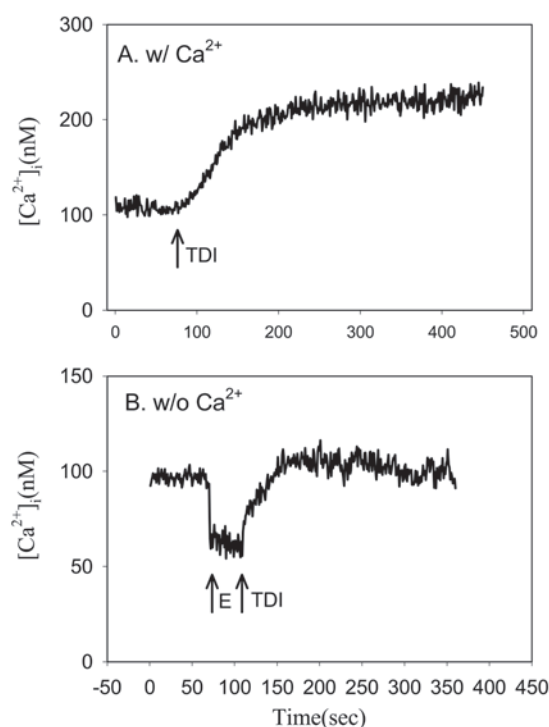


Fig. 1. TDI induced a [Ca²⁺]_i rise in the presence (A) and absence (B) of extracellular calcium in H1355 cells. TDI, at 500 μ M, added to a cuvette ($\uparrow$ TDI) contained fura-2 loaded cells in loading buffer containing 2.2 mM Ca²⁺ (Panel A) or in Ca²⁺ free loading buffer (Panel B). 0.25 mM EGTA was added to chelate the remaining Ca²⁺ shown as $\uparrow$ E.

tive IL-4 transcript levels in TDI-treated and control were adjusted with the results of GAPDH for each experimental condition.

RESULTS

TDI induced an elevation in $[Ca^{2+}]_i$

Freshly prepared TDI induced a rise in $[Ca^{2+}]_i$ in human lung epithelial H1355 cells shown in Fig. 1. In the presence of extracellular Ca^{2+} , a lag time was observed in TDI-induced $[Ca^{2+}]_i$ rise. After 100 sec, a sustained phase occurred at a high level over a long period. In a Ca^{2+} free buffer, freshly prepared TDI also induced an increase in $[Ca^{2+}]_i$ and a lag time were present to achieve the peak. The sustained level was lower compared between without and with extracellular Ca^{2+} . Therefore, the intracellular Ca^{2+} release and extracellular Ca^{2+} influx were both present.

In other cell lines, similar character of TDI inducing a rise in $[Ca^{2+}]_i$ occurred as shown in Fig. 2. 500 μ M TDI induced a net $[Ca^{2+}]_i$ increase of 137 ± 22 nM ($n = 3$) in human T-leukemia Jurkat cells, 70 ± 8 nM ($n = 3$) in human epithelial HeLa cells, 83 ± 16 nM ($n = 26$) in human lung epithelial H1355 cells and 113 ± 17 nM ($n = 21$) in human neuroblastoma SH-SY5Y cells. In all four cell lines, the Ca^{2+} releasing ability of the TDI in the absence of extracellular Ca^{2+} were smaller as comparing with the presence of extracellular Ca^{2+} . In primary isolated bovine adrenal chromaffin cells and human white blood cells, TDI was also capable to induce a rise in $[Ca^{2+}]_i$, and 500 μ M TDI induced a net $[Ca^{2+}]_i$ increase of 80 ± 12 nM ($n = 3$) and 55 ± 5 nM ($n = 3$) in these cells, respectively (Fig. 3).

In the absence of extracellular Ca^{2+} , Fig. 4 shows that 500 μ M TDI induced a net $[Ca^{2+}]_i$ increase of 58 nM in human T-leukemia Jurkat, 44 nM in human epithelial HeLa cells, 31 nM in human lung epithelial H1355 cells and 63 nM in human neuroblastoma SH-SY5Y cells (all $n=3$).

TDI stimulated cytokines release from WBC and Jurkat cells

The cytokine release from human T-leukemia Jurkat cells under TDI stimulation was detected. Significant higher release of IL-4 and IL-6 were found in the supernatants responding to the TDI as early as 30 min but not for IFN- γ , and TNF- α (Fig. 5) when compared with the vehicle (DMSO) only. White blood cells obtained from three donors were used to observe the cytokine release under similar TDI treatments. Fig. 5B shows that the release of cytokines in human white blood cells, only IL-4 was sig-

nificantly higher responding to the short time treatments of TDI, not yet observed in other cytokines, named IL-6, IFN- γ and TNF- α . Similar pattern of IL-4 release induced by TDI stimulation was exhibited in both human T-leukemia Jurkat cells and human white blood cells. The secretion of IL-4 inclines a Th2-driven inflammatory and allergic response might occur after the short term treatments of TDI.

Cytokine expression of Jurkat cells

The mRNA levels of TNF- α , IL-4 and IL-6 were detected after acute TDI treatment. We treat the cells with TDI only for 5 min; then ammonium bicarbonate was added to treat the cells for 25 min. RT-PCR showed that at 3 hr after TDI treatment, the mRNA level of TNF- α was 1.6 times higher than the control with a statistical significance (Fig. 6, $p < 0.05$). The mRNA levels of IL-4 and IL-6 were also higher but no statistical significance was observed. We suggest that an acute stimulation of TDI was enough to promote the cytokine gene expression. However, the mRNA level of TNF- α was increased significantly but IL-4 mRNA level was higher but not significant under the treatment of TDI. We suppose that the increase of IL-4 release might be from the cellular storage but small amount were from the newly synthesized mRNA.

Dose-dependent effects of TDI on IL-4 release

The stimulation of TDI on cytokine secretion was in a dose dependent and a time dependent manner shown in Fig. 7. The release of IL-4 was found in the first 5 min and lasted to 25 min, and was mainly released within the first five min. About 4 folds elevation of IL-4 release was induced by 125 μ M TDI that was suppressed in the presence of BAPTA, an intracellular calcium chelator. The subsequent gene expression was suppressed also (Fig. 6C).

Elevated plasma level of IL-4 in TDI allergic workers

The plasma IL-4 levels were investigated in 30 workers, nine workers were TDI allergic subjects, 6 subjects were non-allergic and 15 allergic subject. IL-4 of TDI-allergic group showed different statistical results with the other two control groups. The IL-4 level of TDI-allergic workers was higher than both the non-allergic group and the non-work related allergic group, and a significant difference was found between TDI-allergic and allergic control workers (Fig. 8). The results implied a Th2 inflammatory cytokine also appeared in human exposed to TDI.

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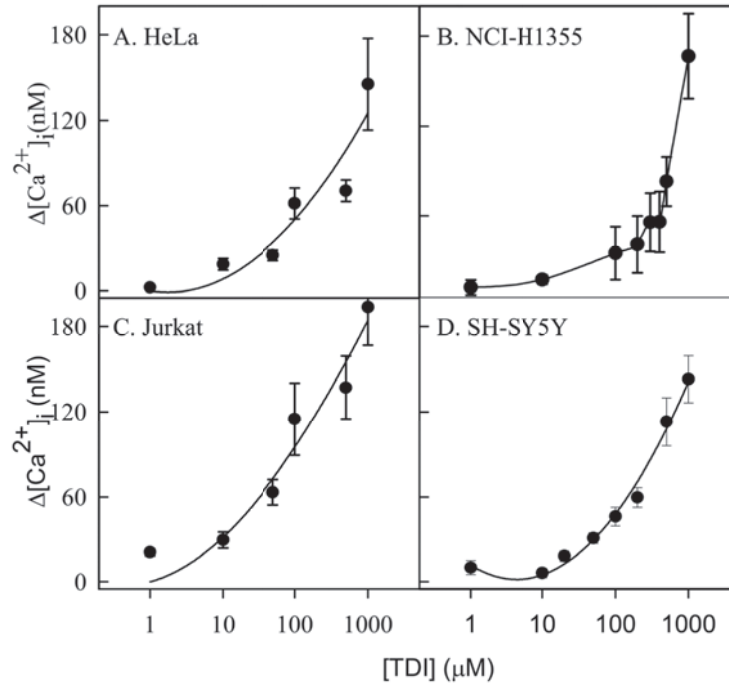


Fig. 2. Concentration-dependent $[Ca^{2+}]_i$ rise induced by TDI in the presence of extracellular Ca^{2+} in various human cell lines. The experiment was preceded as Fig. 1A. Panel A, B, C, D presents the data in the HeLa cells, the H1355 cells, the Jurkat cells and the SH-SY5Y, respectively. The net of $[Ca^{2+}]_i$ rise was calculated by the difference between its basal and its maximal rise after adding TDI 150 sec. The data are mean $\pm$ S.D. of 3-5 experiments (each triplicate).

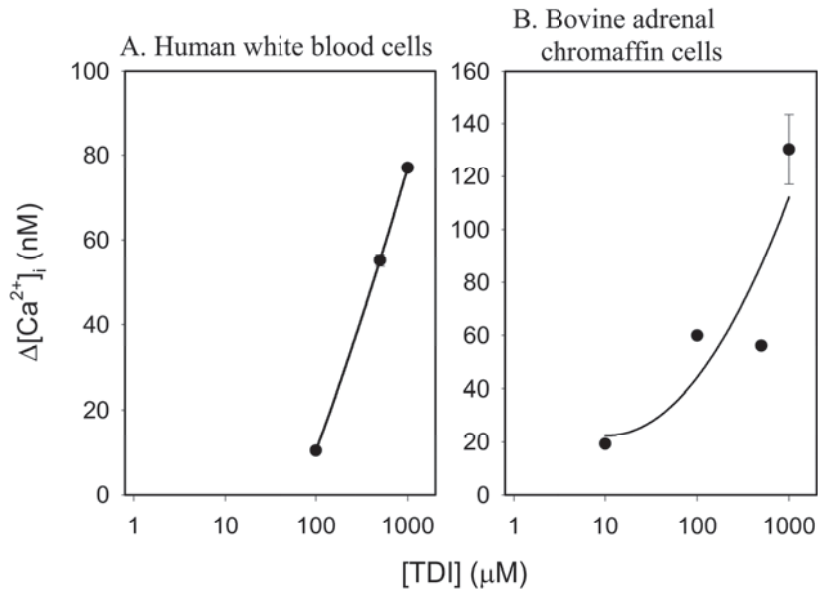


Fig. 3. Concentration-dependent $[Ca^{2+}]_i$ rise induced by TDI in the presence of extracellular Ca^{2+} in native primary isolated cells. The experiment was preceded as Fig. 1A. Panel A, B, presents the data in human white blood cells and bovine adrenal chromaffin cells, respectively. The net of $[Ca^{2+}]_i$ rise was calculated by the difference between its basal and its maximal rise after adding TDI 150 sec. The data are mean $\pm$ S.D. of 3-5 experiments (each triplicate).

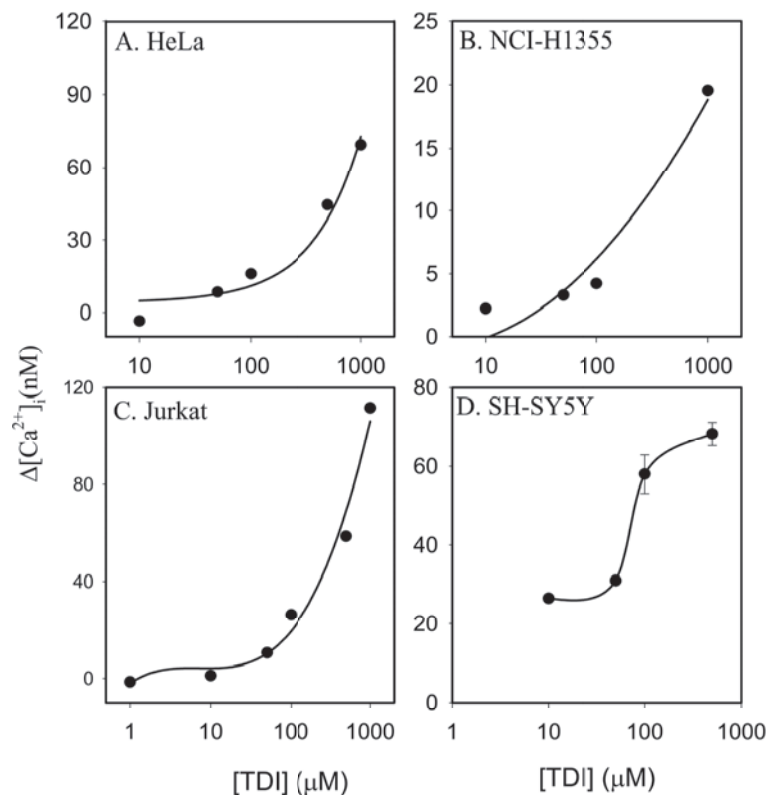


Fig. 4. Concentration-dependent $[Ca^{2+}]_i$ rise induced by TDI in the absence of extracellular Ca^{2+} in various human cell lines. The experiment was preceded as Fig. 1B. Panel A, B, C, D presents the data in the HeLa cells, the H1355 cells, the Jurkat cells and the SH-SY5Y, respectively. The net of $[Ca^{2+}]_i$ rise was calculated by the difference between its basal and its maximal rise after adding TDI 150 sec. The data are mean $\pm$ S.D. of 3-5 experiments (each triplicate).

DISCUSSION

This study mainly illustrates that TDI can induce an elevation of $[Ca^{2+}]_i$ level and its coupled cytokine release, especially IL-4. IL-4 play a crucial role as an inflammatory mediator in allergic asthma via inducing Th2 inflammation and IgE synthesis. This pathway might provide a clue for one of the mechanisms involving TDI-induced asthma, mainly acute responses. Our supporting data listed: 1). TDI induced an elevation in $[Ca^{2+}]_i$ in human cell lines, including T-leukemia Jurkat cells and primary isolated human white blood cells. 2). TDI induced an increase of IL-4 release within 30 min. This increase of IL-4 release might mainly be from its intracellular store and coupled with calcium signaling. 3). TDI acutely induced a mRNA level increase in TNF- α and IL-4, yet not significant in IL-4. We suggest that the IL-4 release within 30 min was mainly from intracellular storage. 4). Higher level of IL-4 in the sera of TDI-allergic workers was also observed. Because the calcium signaling induced

by TDI was transient, therefore, is responsible for TDI acute effects.

The data in several cell lines and native cells in this study suggest that TDI is capable of inducing an elevation of $[Ca^{2+}]_i$ levels, including calcium mobilization from internal storage pools and extracellular calcium influx. This phenomenon was observed both in the presence or absence of external calcium. So, TDI on its own, is responsible for an increase of $[Ca^{2+}]_i$. This suggests that both calcium release and calcium influx together, is responsible for the calcium rise level induced by TDI. However, the depletion of the extracellular calcium resulted in a lower increase in $[Ca^{2+}]_i$ upon the exposure to TDI. This suggests that calcium influx is responsible for the majority of the sustained calcium levels induced by TDI. Furthermore, our data evidenced the existence of TDI-induced $[Ca^{2+}]_i$ rise in epithelial cells (present by HeLa cells), lung cells (present by human lung epithelial H1355 cells), while blood cells (present by human T-leukemia Jurkat) and autonomic nerve (present by human

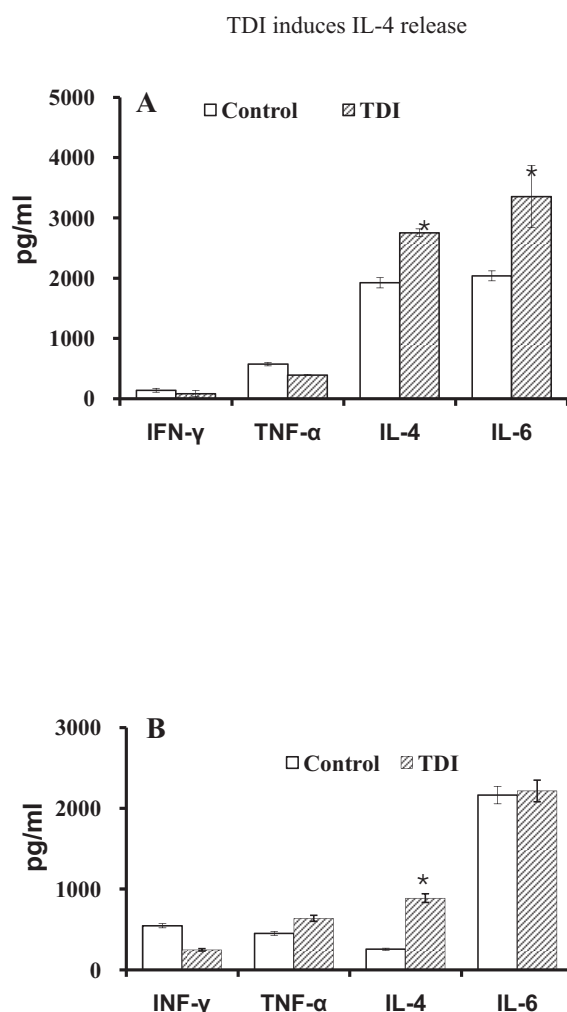


Fig. 5. The cytokines release from Jurkat cells and white blood cell under the TDI treatments. Panel A, B presents the data in Jurkat cells and white blood cell (WBC), individually. The concentrations of IFN- γ , TNF- α , IL-4 and IL-6 in supernatants were measured by ELISA after TDI (1 mM) treatment for 30 min in buffered cellular suspension. The reactivity of TDI was stopped at 5 min by ammonium bicarbonate (0.2 M). Three individual experiments were carried out by using different batch Jurkat cells or using WBC from three donors. Each experiment was triplicate. Bars are means $\pm$ S.D.; * $p < 0.05$, compared to control.

neuroblastoma SH-SY5Y cells). This character of TDI was also evidenced in the native primary isolated cells, bovine adrenal medullary chromaffin cells and human whole blood cells.

Stimulation-secretion coupling triggered by an elevation of $[Ca^{2+}]_c$ level was solidified by many evidences, early findings from neurons that show an elevation of $[Ca^{2+}]_c$ leads neurotransmitter release. Today, stimulation-secretion coupling has been applied in every endocrine, auto-crine release including cytokine release. Recently, natural T cells promptly secrete pro-inflammatory cytokines under activation without prior sensitization were reported, and the activation of those genes through certain pathways important for calcium flux was mentioned. In Jurkat

T cells, interleukin-2 secretion after stimulation was found to be calcium-dependent via relocation of membrane proteins (Podrzywalow-Bartnicka *et al.*, 2007).

In the leukocytes and platelets of patients with bronchial asthma, the level of $[Ca^{2+}]_c$ is higher than those of the healthy people (Chhabra *et al.*, 1999; Efendieva *et al.*, 1988; Kuroda *et al.*, 1995). TDI is known to induce asthma in short-term exposed workers also. Our findings support that TDI is capable to induce $[Ca^{2+}]_c$ rise in primary isolated white blood cells, Jurkat cells and other airway cells. The data on the character of TDI inducing an elevation of $[Ca^{2+}]_c$ in this study may link with the character of asthma people, and illustrates the character of TDI inducing an elevation of $[Ca^{2+}]_c$ might play some roles on its

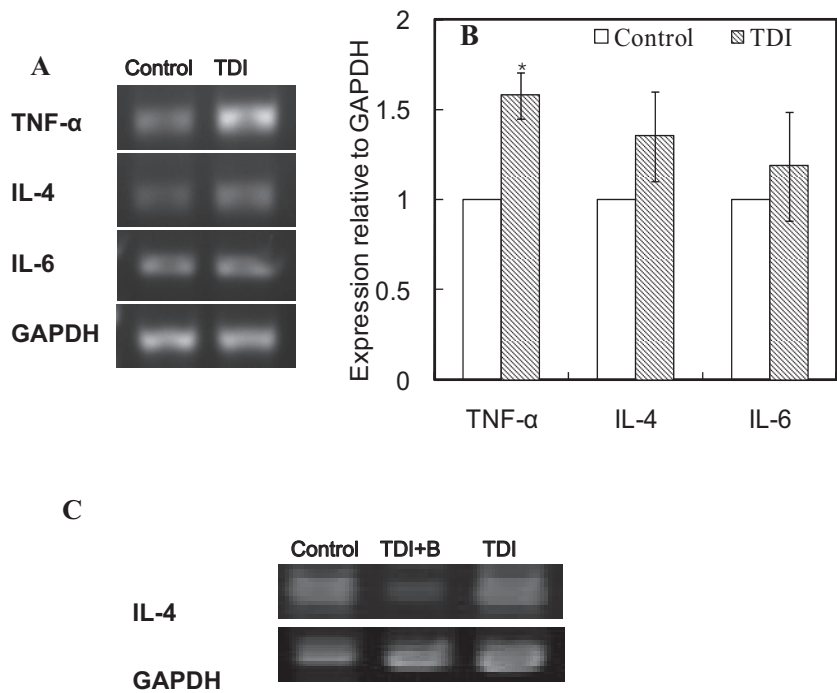


Fig. 6. The cytokine mRNA levels of Jurkat cells treated with TDI. Jurkat cells were stimulated with TDI (1 mM) for 30 min and resuspended in culture medium for 3 hr. The reactivity of TDI was stopped at 5 min by ammonium bicarbonate (0.2 M). Panel A shows the mRNA levels of TNF- α , IL-4 and IL-6 analyzed by semi-quantitative RT-PCR using specific primer listed in the Materials and Methods. GAPDH is the internal control in this experiment. Panel B shows the relative expression levels which were calculated by the ratio of individual genes compared to GAPDH. Bars are means $\pm$ S.D.; * $p < 0.05$, compared to control. Panel C shows the mRNA levels of IL-4 in the presence of 1 mM TDI (shown as TDI), 1 mM TDI with 5 μ M BAPTA (shown as TDI+B) and vehicle (shown as control).

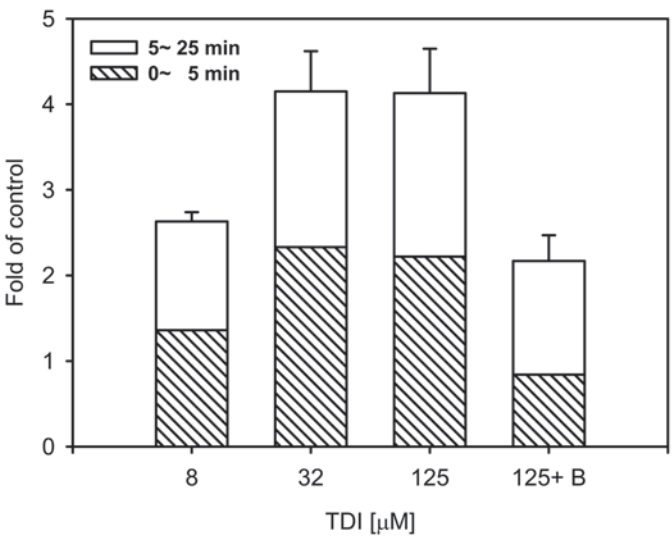


Fig. 7. The time and dose effects of TDI on the IL-4 release from Jurkat cells. IL-4 release from the cells stimulated by TDI within 0-5 min (striated bars) and 5-25 min (open bars). Different concentrations of TDI were used as shown in X scale and 125 + B presents the treatment of 125 μ M TDI with 5 μ M BAPTA.

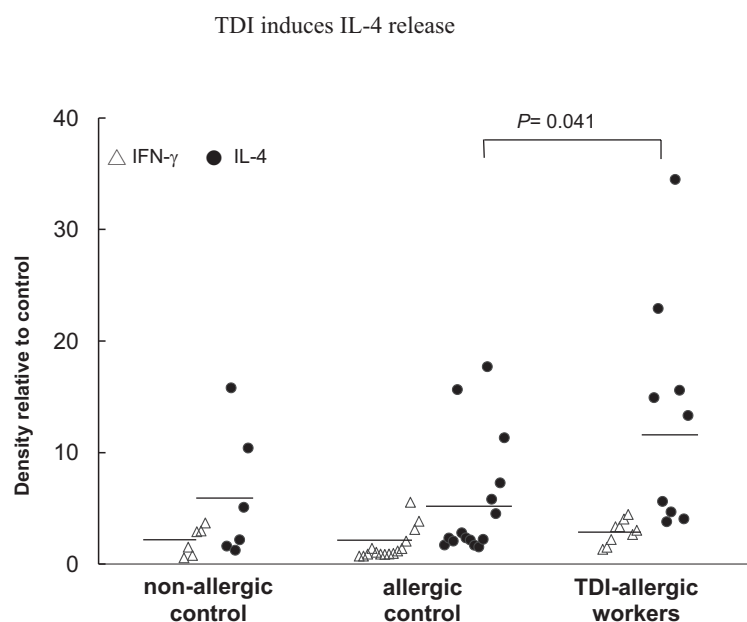


Fig. 8. Comparison of plasma levels of IFN- γ and IL-4 among the subjects of TDI-allergy, non-allergic and allergic control by ELISA. Statistic differences were analyzed by one-way ANOVA. Results are expressed as dots for each tests and the mean as horizontal lines. The solid circles represent the relative density value of IL-4 to the isotype controls, and the open triangle for the INF- γ . The content of IL-4 in the sera of TDI-allergic workers is higher than both of the non-allergic and the non-TDI allergic control. Significant difference was only found between TDI-allergic workers and allergic workers not related to TDI ($P = 0.041$).

induction of asthma.

TDI-induced asthma leads to neutrophil movement and cell-mediated immune responses (Bentley *et al.*, 1992; Finotto *et al.*, 1991; Mapp *et al.*, 1994a). After exposure to TDI, inflammatory cells including eosinophils and mast cells are degranulated in the airway mucosa (Saetta *et al.*, 1992). During activation, granulocytic cell changing intracellular free calcium levels have been proved to be associated with degranulation, which causes cell releasing storage from granules, such as eosinophils and mast cells releasing their cytokines (Liu *et al.*, 2006b). In similar ways, a series pathway can be predicted that the cells present in airway can be triggered after an elevation of $[Ca^{2+}]_i$, which is induced by TDI during exposure.

In isocyanate-induced asthma characterized by airway inflammation, TNF- α is critical to the inflammation process (Matheson *et al.*, 2002). According to its multiple and central roles in TDI-induced asthma, TNF- α , may influence both nonspecific inflammatory processes and specific immune events. TNF- α is likely a central mediator of airway inflammation and bronchial hyper responsiveness in asthma, with high levels in bronchoalveolar lavage fluid (Van Oosterhout and Nijkamp, 1993). The expression of IL-4 protein was increased only in the asthmatic subjects tested after 48 hr exposure to TDI (Maestrelli *et al.*, 1997). Animal models indicated that

Th2 cytokines appeared after TDI stimulation. In the experiment of C57BL/6J mice, after TDI challenge, histological changes in the trachea of wild-type mice were accompanied by increased mRNA expression of IL-4 and TNF- α compared with the athymic mice (Matheson *et al.*, 2001). Short term exposure of TDI vapor resulted in airway symptoms in rats, and significant production of IL-4 in the airways of the exposed group (Kouadio *et al.*, 2005). In airways, because of high humidity, most of air-born TDI are lost quickly (Tsai *et al.*, 2006) and might solute in mucous fluid within several minutes after entering. Follow-up reactive events might occur with proper molecules possessing hydrophobic moieties appearing in airway fluids. Local micro-environments might allow similar reaction as calcium elevation induced secretion exists. Our results provide a possible way explaining the elevated levels of TNF- α and IL-4 in bronchoalveolar lavage fluid induced by short term-TDI exposure.

We conclude that TDI directly induces an elevation in $[Ca^{2+}]_i$ in cell lines and primary cultured cells as well as IL-4 release from Jurkat cells and human white blood cells. Our results illustrate that IL-4 might be an early factor to crank up the inflammatory responses, and the release of it was coupled with an elevation in $[Ca^{2+}]_i$ induced by TDI. This pathway related to TDI was rapidly occurred, thus the acute inflammation induced by TDI

can be explained, especially for asthma.

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