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Toll-like receptor 2 is upregulated by hog confinement dust in an IL-6 dependent manner in the airway epithelium

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Abstract

Hog confinement workers are at high risk to develop chronic bronchitis as a result of their exposure to organic dust. Chronic bronchitis is characterized by inflammatory changes of the airway epithelium. A key mediator in inflammation is Toll-like receptor 2 (TLR2). We investigated the role of TLR2 in pulmonary inflammation induced by hog confinement dust. Normal Human Bronchial Epithelial Cells (NHBE) were grown in culture and exposed to hog confinement dust extract. Hog confinement dust upregulated airway epithelial cell TLR2 mRNA in a concentration and time-dependent manner using real-time PCR. There was a similar increase in TLR2 protein at 48 hours as shown by Western blot. TLR2 was upregulated on the surface of airway epithelial cells as shown by flow cytometry. A similar upregulation of pulmonary TLR2 mRNA and protein was shown in a murine model of hog confinement dust exposure. Hog confinement dust is known to stimulate epithelial cells to produce IL-6. In order to determine whether TLR2 expression was being regulated by IL-6, the production of IL-6 was blocked using an IL-6 neutralizing antibody. This resulted in attenuation of the dust-induced upregulation of TLR2. To further demonstrate the importance of IL-6 in the regulation of TLR2, NHBE were directly stimulated with recombinant human IL-6. IL-6 alone was able to upregulate TLR2 in airway epithelial cells. Hog confinement dust upregulates TLR2 in the airway epithelium through an IL-6 dependent mechanism.

Keywords

toll-like receptor 2; chronic bronchitis; hog confinement dust; IL-6

Introduction

Pulmonary disease is a well-established occupational hazard of production agriculture. (6,10) Hog confinement workers have been shown to develop chronic bronchitis and asthma as a result of their exposure to organic dust (9,22). Hog confinement dust has been shown to incite inflammation of the airway epithelium (18). Airborne microorganisms are present in the dust and are also thought to contribute to the inflammation (17).

The lung's first line of defense against airborne microorganisms is the airway epithelium. The airway epithelium expresses Toll-like receptors (TLRs), which play a critical role in triggering the inflammatory response directed against microbial insults. TLRs recognize invading microbial pathogens through pathogen associated molecular patterns (PAMPs). PAMPs are

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highly conserved microbial components such as lipopolysaccharide, peptidoglycan and flagellin. There are ten known human toll-like receptors and each recognizes a different PAMP (1).

Toll-like receptor 2 (TLR2), which recognizes peptidoglycan, LTA (20) and zymosan (8,19), is expressed in airway epithelium and is a key mediator in lung innate immunity. When TLR2 is activated, it initiates the cellular inflammatory response to gram-positive microbial invasion. In this study, we investigated the effect of hog confinement dust exposure on expression of TLR2 mRNA and protein in an *in vitro* model of airway epithelial cells and in an *in vivo* murine model of hog confinement dust exposure. We had previously shown that IL-6 is rapidly secreted by the airway epithelium in response to hog confinement dust stimulation (18). This led us to hypothesize that IL-6 may be a critical hog confinement dust mediator that stimulates TLR2 expression in the airway epithelium.

Materials and Methods

Cell Culture

Normal human bronchial epithelial cells (NHBE) (Lonza (Walkersville, MD: Lot numbers: 2F1341, 2F1678, 4F1499), were grown in submerged culture using serum free Bronchial epithelial basal media (BEBM) (Lonza), supplemented with BEGM SingleQuots (Lonza). The cells were cultured in plates coated with 1% Vitrogen solution. (Cohesion, Paolo Alto, CA). Cells were maintained in a 37°C incubator with 5% carbon dioxide. Cells were fed every 24-48 hours until they were approximately 80% confluent, at which time experiments were performed. Experiments were performed on cells that were passage 2-6. Morphology of the cells was carefully observed prior to experiments. Cells demonstrating a squamous morphology were not utilized.

Hog Confinement Dust Extract preparation

Hog confinement dust was collected from settled dust at 2 separate hog confinement buildings. Both dusts were used in experiments with similar results. Hog dust extract was prepared as previously described (12). Hog dust extract was prepared by mixing 10 grams of the dust and 10ml Hank's Balanced Salt Solution without calcium. The mixture was incubated at room temperature for 1 hour. The mixture was centrifuged for 10 minutes and the supernatant was decanted and re-centrifuged for 10 minutes. The supernatant was again decanted, sterile filtered, and used immediately.

RNA extraction

NHBE were grown to 60-70% confluency then exposed to various concentrations of hog dust extract (HDE) 1%, 2.5%, 5% at various time points 1,6,24 hours. Cell monolayers were rinsed twice in HEPES solution, then trypsinized and stored in RNA Later (Applied Biosystems, Foster City, CA) until RNA extraction could be performed. RNA was extracted and genomic DNA was removed using the Magmax 96 kit (Applied Biosystems) according to the manufacturer's instructions. Concentration and purity of the RNA was determined using the NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). All RNA samples had a A260/280 ratio of 1.9-2.0.

Real-time RT-PCR

First strand cDNA was synthesized using 100ng of template RNA and the Taqman reverse transcription kit (Applied Biosystems, Foster City, CA). Reactions were prepared containing: 1X TaqMan RT Buffer, 5.5mM Magnesium Chloride, 500µM of each dNTP, 2.5µM Random hexamers, 0.4 U/µl RNase Inhibitor, and 1.25 U/µl of MultiScribe Reverse Transcriptase.

Samples were incubated in a thermocycler at 25°C for 10 minutes, then 48°C for 30 minutes, then 95°C for 5 minutes.

Real-time PCR reactions were prepared using 1X TaqMan Master Mix (Applied Biosystems), human TLR2 primers and probes (Applied Biosystems Hs00152932_m1). Ribosomal RNA was used as an endogenous control. PCR was performed using ABI PRISM 7700 Sequence detection system (Applied Biosystems). Reactions underwent 50°C for 2 minutes, 95°C for 10 minutes then 40 cycles of 95°C for 15 sec and 60°C for 1 minute. Each reaction was carried out in duplicate. For relative comparison of TLR2 to ribosomal RNA endogenous control, we analyzed the Ct value of real-time PCR data with delta-delta Ct method (11).

Western blot analysis

NHBE cells were grown to 60-70% confluency then exposed to 5% HDE for 48 hours. Cells were lysed on ice for 20 minutes with lysis buffer consisting of: 10mM Tris, 150mM NaCl, 3mM EDTA, 100μM leupeptin, 10μM aprotinin, 20 μg/ml of soybean trypsin inhibitor, 1mM PMSF, 5mM benzamidine, 1% Triton X-100. The concentration of protein in the samples was determined by spectrophotometry. Each well was loaded with 25μg of total protein prior to polyacrylamide gel electrophoresis and electro-blotting to nitrocellulose. Rabbit anti-human TLR2 (H-175) antibody (SantaCruz, Santa Cruz, CA: sc-10739) and rabbit anti-human TLR4 (H-80) (SantaCruz: sc-10741) were used in a 1:400 dilution. Goat anti-Rabbit horseradish peroxidase-conjugated secondary antibody (Millipore, Temecula, CA) was used in a 1:10,000 dilution. The blots were imaged using the Visualizer™ Western Blot Detection Kit (Millipore) and exposed to x-ray film. Beta-actin loading controls were performed to ensure loading of equal amounts of protein. The blots were scanned and densitometry was performed using ImageJ software.

Fluorescent Activated Cell Sorting (FACS)

NHBE were grown in submerged culture to 60-70% confluency and exposed to 1-5% HDE for 48 hours. Cells were trypsinized and washed. They were incubated with mouse anti-human TLR2 [TL2.1] (Abcam, Cambridge, MA: ab-9100) for 1 hour, washed and incubated for 1 hour with an Alexa 647 conjugated secondary antibody (Invitrogen, Carlsbad, CA). Cells were washed twice and fixed with 1% paraformaldehyde in phosphate buffered saline (pH 7.4). Analysis by fluorescence activated cell sorting (FACS) was performed using a FACSCaliber flow cytometer using BD CellQuest software (Becton Dickinson, San Jose, CA) The Mean Fluorescence Intensity (MFI) was reported for 10,000 events for each experimental condition.

Mouse exposure to HDE

All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the Omaha Veterans Affairs Medical Center and the University of Nebraska Medical Center.

Female C57BL/6 mice, age 6-8 weeks, (Charles River, Wilmington, MA) were acclimated for 1 week after shipping. During this time, they were group housed and fed commercial rodent chow and water *ad libitum*. The mice were then randomly assigned to a treatment group: No handling, PBS instillation, 1%, 5%, or 12.5% HDE instillation. There were 4 mice in each group. Mice assigned to an instillation group received daily nasal instillations for 1 week.

The procedure for nasal instillation of HDE is based on a previously established model using cigarette smoke extract (5,14). The instillations were carried out as follows: prior to nasal instillations, the mice were anesthetized with isofluorane. Each mouse was held vertically while 50 μl of PBS or HDE was placed at the opening of the nare. The mice were held in the vertical position until the solution was inhaled through the nasal cavity and into the lungs. They were

then monitored until awake and moving around normally following the treatment. No mice exhibited respiratory distress. All mice were weighed throughout the instillation time course. No weights of any group of mice were significantly different from another group.

Mice were sacrificed at the end of the experiment and the lung tissue was flash frozen. This tissue was homogenized and RNA extraction was performed using the MagMax kit as described above. The real-time PCR protocol was also the same, substituting mouse TLR2 primers and probe (Applied Biosystems Mm00442346_m1). Protein was extracted from the lung homogenate and Western blots were performed using the same protocol and antibodies described above.

IL-6 blocking experiments

To study the role of IL-6 in HDE-induced TLR2 expression, NHBE were pre-incubated with or without IL-6 neutralizing antibody (R&D AB-206-NA, Minneapolis, MN) at 5µg/ml for 1 hour. Cells were then stimulated with and without 1% HDE for 24 hours. The anti-IL-6 antibody remained in the media throughout the 24-hour stimulation. Cells were treated with goat IgG (Sigma, St. Louis, MO) at the same concentration to assess for non-specific binding. Cell layers were harvested and RNA was extracted as described above.

Recombinant human IL-6 experiments

NHBE were exposed to 5-20ng of rhIL-6 (R&D) for 24 hours. These concentrations of IL-6 correspond to the amount of IL-6 that epithelial cells typically produce in response to HDE. Cell layers were harvested and RNA was extracted as described above.

LDH assay

Cell viability was determined using an LDH assay. We used the “In vitro toxicology assay kit, lactate dehydrogenase based” (Sigma) according to the manufacturers instructions. It was performed on supernatants of cells exposed to varying concentrations of HDE. There was no cell death at the concentrations of HDE used in this paper.

Statistics

Each quantitative experiment was repeated a minimum of 3 times with different lot and passage numbers of NHBE. Data are presented as mean values $\pm$ SEM. Statistical significance was determined using student's t-test. Differences were considered statistically significant at the 95% Confidence Interval if $P \leq 0.05$.

Results

HDE increases TLR2 mRNA in a time- and concentration-dependent manner in airway epithelial cells

To determine the effect of HDE on expression of TLR2, NHBE were exposed to 5% HDE for 1, 6, 24 hours. We observed a statistically significant increase in TLR2 mRNA expression at each time point between 1-24 hours. (Figure 1A). Cell viability was documented by insignificant LDH release at all time points.

NHBE were also exposed to 1-5% HDE for 24 hours, the time of peak TLR2 mRNA expression. TLR2 mRNA was upregulated in a concentration-dependent manner by HDE. There was a statistically significant increase in TLR2 mRNA expression with each concentration of HDE, with a maximum increase detected at 5% (Figure 1B). Concentrations of HDE greater than 5% did not cause further increase in TLR2 mRNA expression.

HDE increases TLR2 protein, which is expressed on the cell surface

To determine whether HDE also induced translation of TLR2 mRNA into protein, we performed Western blots and FACS. In these experiments, NHBE were exposed to 5% HDE for 48 hours, and Western blots were performed. Compared to control, there was a 4.0 fold increase in TLR2 protein in cells exposed to 5% HDE (Figure 2A and 2B). Because endotoxin is important in the agricultural setting, we also assessed HDE's effect on TLR4. We saw no difference in TLR4 in stimulated versus unstimulated cells (Figure 2C and 2D).

To determine whether TLR2 was transported to the cell surface after synthesis, FACS was performed. FACS revealed a concentration dependent increase of TLR2 protein expressed on the cell surface, with a 1.6 fold increase with 1% HDE and a 3.8 fold increase with 5% HDE (Figure 3). An isotype control showed negligible staining. HDE alone did not cause any autofluorescence.

HDE upregulates TLR2 in a murine model

Because our data revealed that HDE upregulates TLR2 in airway epithelium *in vitro*, we hypothesized that HDE upregulates TLR2 *in vivo*. To test this hypothesis, mice were exposed to intranasal PBS or varying concentrations of HDE for 1 week. RNA was extracted from whole lung homogenate and real-time PCR was performed. Mice exposed to 5% and 12.5% HDE had statistically significant increases in TLR2 mRNA, which correlated with increasing HDE concentration (Figure 4A). Likewise, there was a corresponding increase in TLR2 protein in the lung homogenates of HDE-treated mice as detected by Western blot (Figure 4B and 4D).

IL-6 mediates the upregulation of TLR2 by HDE

To determine whether IL-6 was participating in the modulation of TLR2, NHBE were preincubated with and without anti-IL-6 antibody for 1 hour, then stimulated with or without 1% HDE. As in previous experiments, we saw a 3.5 fold increase in TLR2 mRNA in cells stimulated with 1% HDE. The upregulation of TLR2 was greatly attenuated when the cells were pre-incubated with anti-IL-6. Compared to control, unstimulated cells pretreated with IL-6 also had a statistically significant ($p=0.01$) decrease in baseline TLR2 mRNA production (Figure 5A). No change was observed in cells treated with an isotype control antibody at the same concentration (data not shown).

To further investigate the role of IL-6 in modulating TLR2, NHBE were directly stimulated with 5-20ng/ml of recombinant IL-6 (rhIL-6) for 24 hours. This represents the range of IL-6 concentrations detected in supernatants of cells stimulated with HDE. We found that rhIL-6 stimulates TLR2 mRNA production in a concentration-dependent manner (Figure 5B).

Discussion

In these experiments, we have demonstrated that hog confinement dust upregulates TLR2 in a concentration- and time-dependent manner in the airway epithelium. We have shown that an increase in TLR2 mRNA leads to an increase in synthesis of TLR2 protein and that this increase in TLR2 occurs on the airway epithelial cell surface. This upregulation of TLR2 not only occurs *in vitro*, but importantly, it occurs *in vivo* using a murine model of inhaled hog confinement dust exposure.

We have shown TLR2 is upregulated in the airway epithelium by exposure to organic dust. Organic dusts have been shown to upregulate TLR2 in bone marrow dendritic cells (3). However, our finding is novel in airway epithelial cells. Importantly, this upregulation of TLR2 is dependent on the dust-induced production of IL-6 by the airway epithelium. The exact component(s) in hog dust that trigger TLR2 expression are not known.

Hog confinement dust is a complex mixture composed of many substances including bacterial products such as peptidoglycan and lipopolysaccharide. It also contains particulate matter from grain particles and fecal matter (4). It is possible that many of these substances contribute to the production of IL-6 and subsequent upregulation of TLR2 to some degree. For instance, peptidoglycan is known to increase IL-6 production. Particulate matter has also been shown to upregulate TLR2 in airway epithelial cells. (2) Coarse particulate matter ($>2.5\mu\text{m}$) has been shown to be the most potent stimuli. However, it is not likely to be a major contributor to the upregulation of TLR2 in our experiments, because coarse particulate matter was removed in our sterile filtering process. Another component of the dust that could possibly regulate Toll-like receptor expression is lipopolysaccharide (LPS), a component of the gram-negative cell wall. LPS is known to signal through TLR4. *In vitro*, TLR4 is not thought to play a large role in airway epithelial cell inflammation. Airway epithelial cells lack MD-2, an essential cofactor for TLR4 signaling (15,16). However, clinically, LPS has been associated with respiratory symptoms in hog confinement workers (21). Because of this correlation between LPS and respiratory symptoms, we also looked at the role of LPS and TLR4. In experiments stimulating NHBE with HDE, we did not see any upregulation of TLR4 mRNA (data not shown) or protein (Figure 2B). Likewise, removing endotoxin from the HDE did not diminish the upregulation of TLR2 (Data not shown). Therefore, it is not likely that LPS is playing a large role in the upregulation of TLR2 that we have observed.

We were also able to show that HDE upregulates TLR2 in an *in vivo* model of exposure. In mice exposed to hog confinement dust intranasally for a week, HDE upregulates TLR2 in the lung. This was true both for TLR2 mRNA and protein. Because we performed our RT-PCR and Western blots on total lung homogenates we cannot conclude that the source is solely the airway epithelium although we speculate that is a major contributor to whole lung TLR2 expression.

Perhaps our most interesting finding is that IL-6 is an essential modulator in the regulation of TLR2 in the airway epithelium. Other cytokines have been shown to regulate TLR2 in airway epithelial cells. Tumor necrosis factor- α (TNF α) and interferon- γ have been shown to synergistically enhance expression of TLR2 in the airway epithelium (7). In macrophages, IL-12, IL-15, IL-1 β , TNF α , and interferon- γ have been shown to increase TLR2 mRNA (13). It is not entirely unexpected then that IL-6, an important inflammatory cytokine in the airway epithelium would participate in mediating TLR2 expression. We were able to demonstrate the importance of IL-6 in the regulation of TLR2 in 2 ways. First, we showed that blocking IL-6 production dramatically attenuates the upregulation of TLR2 both at baseline and after HDE stimulation. In addition, stimulation of NHBE with recombinant human IL-6 alone led to an increase in TLR2 mRNA that is similar to what we see with HDE stimulation. The exact mechanism of how IL-6 upregulates TLR2 is unknown. We speculate that IL-6 binds to its receptor and activates the JAK/STAT pathway. This could induce the MAPK cascade, leading to the production of transcription factors, which cause increased transcription of TLR2.

In vivo, the role of IL-6 in the regulation of TLR2 may be even more important. Monocytes and macrophages present in the lung frequently produce IL-6 in response to infection early in the inflammatory process, compared to airway epithelium. This early IL-6 production may prime the airway epithelium for a more vigorous response by initiating an earlier upregulation of TLR2. Further studies into the role of IL-6 in the regulation of TLR2 *in vivo* are necessary.

Although these experiments show that it is likely that IL-6 plays a role in the upregulation of TLR2, we are not able to conclude that this is the only cytokine that helps to regulate TLR2. Other cytokines that are known to enhance TLR2 expression such as TNF α and interferon γ may also play a role (7). However, hog confinement dust, even at high concentrations, does not stimulate production of large amounts of these cytokines. Hog confinement dust does

stimulate airway epithelial cells to produce large amounts of IL-8 (18). When we blocked the production of IL-8, this did not diminish TLR2 mRNA (data not shown). Likewise, when we stimulated cells with recombinant IL-8, we did not see an increase in TLR2 mRNA (data not shown). Based on this, we do not feel it is likely that IL-8 plays a large role in the regulation of TLR2 in airway epithelial cells. It is possible that other cytokines are also produced and may play a role in TLR2 regulation. Further research is necessary to determine this.

Taken as a whole, this series of experiments demonstrates that hog confinement dust upregulates TLR2 in the airway epithelium through an IL-6 dependent mechanism. This upregulation of TLR2 likely leads to increased inflammation in the airway epithelium, which could lead to, or exacerbate diseases of the airway such as chronic bronchitis. TLR2, along with IL-6, likely plays a role in the development of airway disease in hog confinement workers.

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Figure 1.

TLR2 mRNA is upregulated by hog confinement dust in a time- and concentration-dependent manner in airway epithelial cells. A. NHBE were incubated with (solid line) or without (dashed line) 5% HDE at 1,6, and 24 hours. Real-time PCR for TLR2 was performed. Data is expressed as fold change in TLR2 compared to control $\pm$ SEM. (n=4). B. NHBE were incubated with varying concentrations of HDE (solid line) or without (dashed line) for 24 hours. Real-time PCR for TLR2 was performed (n=4).

Figure 2.

TLR2 protein is upregulated by hog confinement dust in the airway epithelium. A. NHBE were incubated with or without 5% HDE. Cells were lysed and Western blot for TLR2 is shown.

TLR2 was detected at its expected molecular mass of, 86 kD. This is a representative blot of 4 separate experiments. B. Densitometry was performed showing a greater than 4 fold increase in TLR2 protein expressed in the HDE-treated cells compared to the media-treated cells. C. In

the same experiments we also probed for TLR4. D. Densitometry showed no increase in TLR4 in response to HDE.

Figure 3.

TLR2 receptors are upregulated on the plasma membrane by hog confinement dust in the airway epithelium. NHBE were incubated with media only, 1% HDE and 5% HDE for 48 hours. FACS staining for TLR2 was performed. A. Representative histogram of 3 separate experiments. Open histogram represents isotype control and solid histogram represents TLR2 staining. B. Summary bar graph shows an increasing Mean Fluorescence Intensity (MFI) with increasing concentrations of HDE.

Figure 4.

TLR2 mRNA and protein is upregulated in the lung of a murine model of hog confinement dust exposure. A. Mice (n=4/group) were exposed to PBS, 1% HDE, 5% HDE and 12.5% HDE daily for 1 week. RNA was extracted from total lung homogenate and real-time PCR for TLR2 was performed. Data is expressed as mean fold change from mice with no handling $\pm$ SEM. B. Western blot for TLR2 from a representative mouse lung from each group demonstrates the 86kDa TLR2 protein band is upregulated in response to increasing concentrations of instilled HDE. C. Beta Actin loading control lane. D. Ratio of the densitometry of the TLR2 Western blot to the Beta Actin loading control demonstrates a 4-5 fold increase in TLR2 protein in response to mouse lung instillation with 1-12.5% HDE.

Figure 5.

IL-6 plays an important role in the upregulation of TLR2 in airway epithelial cells. A. NHBE were pre-incubated with or without anti-IL-6 for 1 hour then stimulated with or without 1% HDE for 24 hours. Real-time PCR for TLR2 was performed. Data expressed as fold change in TLR2 normalized to ribosomal RNA $\pm$ SEM. (n=4). B. NHBE were stimulated for 24 hours with varying concentrations of recombinant human IL-6. Real-time PCR for TLR2 was performed (n=4) showing that 20ng/ml of rhIL-6 significantly ($p=0.01$) increases TLR2 mRNA expression.