Acrolein Induction of Oxidative Stress and Degranulation in Mast Cells

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ABSTRACT: Increases in asthma worldwide have been associated epidemiologically with expanding urban air pollution. The mechanistic relationship between airway hyper-responsiveness, inflammation, and ambient airborne triggers remains ambiguous. Acrolein, a ubiquitous aldehyde pollutant, is a product of incomplete combustion reactions. Acrolein is abundant in cigarette smoke, effluent from industrial smokestacks, diesel exhaust, and even hot oil cooking vapors. Acrolein is a potent airway irritant and can induce airway hyper-responsiveness and inflammation in the lungs of animal models. In the present study, we utilized the mast cell analog, RBL-2H3, to interrogate the responses of cells relevant to airway inflammation and allergic responses as a model for the induction of asthma-like conditions upon exposure to acrolein. We hypothesized that acrolein would induce oxidative stress and degranulation in airway mast cells. Our results indicate that acrolein at 1 ppm initiated degranulation and promoted the generation of reactive oxygen species (ROS). Introduction of antioxidants to the system significantly reduced both ROS generation and degranulation. At higher levels of exposure (above 100 ppm), RBL-2H3 cells displayed signs of severe toxicity. This experimental data indicates acrolein can induce an allergic inflammation in mast cell lines, and the initiation of degranulation was moderated by the application of antioxidants.

INTRODUCTION

Acrolein, a major constituent of combustion products and tobacco smoke, is best known as a carcinogen and produces protein and DNA adducts in high concentration exposures (Liu et al., 2010; Zhang et al., 2011). For example, acrolein induces G to T and G to A mutations forming mutagenic α- and γ-hydroxy-1, N(2)-cyclic propano-2′-deoxyguanosine adducts. These adducts enhance oxidative DNA damage-induced mutagenesis, inhibit DNA repair proteins (Wang et al., 2012), and may contribute to p53 mutations in lung cancer (Feng et al., 2006).

Asthma is a disease on the rise around the world. Exacerbations of asthmatic episodes are associated with increases in air pollution through an abundance of epidemiological data (Samoli et al., 2011). Asthma is typically characterized by a chronic state of inflammation combined with hyper-responsive airway constriction (Hargreave and Nair, 2009). However, the physiological relationship between asthma and air pollution remains elusive.

Acrolein is also a known respiratory irritant (Ghilarducci and Tjeerdema, 1995). Airborne aldehydes have been implicated as contributing factors to the increase in asthma and allergic disease in world urban populations (Leikauf,
method....

Airway mast cells release inflammatory mediators, such as histamine, cytokines, and leukotrienes, responsible, in part, for the initiation of allergic inflammation (Fireman, 2003). It is thought that oxidative stress may contribute to the release of these mediators (Li et al., 2003). Previously, we have shown that sodium sulfite, the soluble form of sulfur dioxide, induces intracellular oxidant stress and degranulation in mast cells (Collaco et al., 2006).

In this study, we sought to investigate the effects of acrolein on mast cell function. We hypothesized that acrolein acts directly on mast cells to induce the release of inflammatory mediators and production of intracellular reactive oxygen species (ROS).

**METHODS**

**Reagents**

Bovine serum albumin (BSA), N-acetyl-1-cysteine (NAC), tetramethylthiourea (TMTU), diphenyleneiodonium (DPI), 2',7'-dichlorofluorescein diacetate (DCFH-DA), Acrolein (C3H4O), and mouse IgE anti-dinitrophenyl (DNP-IgE) antibodies were purchased from Sigma-Aldrich (St Louis, MO). Dinitrophenyl coupled to BSA (DNP) was purchased from Biosearch Technologies (Novato, CA). Dulbecco modified eagle medium (DMEM), fetal calf serum (FCS), streptomycin-penicillin, and Hanks balanced salt solution (HBSS) were purchased from Gibco BRL (Grand Island, NY).

**Cell Culture**

RBL-2H3 cells were obtained from American Type Cell Collection (ATCC) and cultured in DMEM supplemented with 10% FCS, 100 IU/mL penicillin, and 100 μg/mL streptomycin (Gibco BRL, Grand Island, NY) at 37°C in a humidified atmosphere with 5% CO2.

**Mast Cell Degranulation**

As described in a recent review (Rashid et al., 2012), RBL-2H3 cells were established as an efficient and reliable alternative to the wild-type mast cell in inflammation research. These experiments were performed as described previously (Collaco et al., 2006). Briefly, RBL-2H3 cells were plated at 1 x 10^4 per well in a 96-well plate in DMEM supplemented with 5% FCS and 1 μCi/mL of [H3]serotonin overnight. Cells were then washed with HBSS, followed by application of experimental conditions. After incubation, supernatants were harvested, and any cells present were removed by centrifugation. Total cellular content of serotonin was quantified in the supernant of cells lysed with Triton X100. Radioactivity was measured by scintillation spectroscopy (1450 MicroBeta; PerkinElmer, Fremont, CA) and the percentage release calculated: (experimental–spontaneous)/(total-spontaneous). For calcium depletion experiments, cells were labeled with [H3]serotonin and then washed and assayed with calcium free HBSS plus 1 mM EDTA.

**Determination of Intracellular ROS Generation**

RBL-2H3 cells were washed with phosphate-buffered saline (PBS) before loading with 50 μM DCFH-DA in PBS for 20 min at 37°C. The cells were then washed with PBS, incubated in experimental conditions and immediately placed into an automated fluorometer (FLUOstar Optima, BMG Labtech, Durham, NC), with an excitation wavelength of 480 nm and emission of 530 nm. The antioxidants, TMTU (50 μM) and NAC (10 mM) were prepared in PBS and mixed with and without acrolein before adding to wells. In experiments using DPI, cells were incubated with 100 μM DPI for 30 min at 37°C, followed by two washes with PBS before DCFH-DA loading. The inhibitors themselves did not induce fluorescence. Results were calculated by subtracting spontaneous fluorescence in wells containing PBS alone and are expressed in relative fluorescence units.

**Cellular Viability Measurements**

Cell viability was evaluated using several methods. Propidium iodide (PI) (Calbiochem, San Diego, CA) staining was used to assess cellular membrane integrity. Cells were incubated for 30 min with acrolein. The cells were then harvested with trypsin plus 0.1% EDTA, washed twice with PBS with 0.5% BSA, and stained with PI (1 μg/mL) in PBS with 0.5% BSA. Fluorescence was evaluated by flow cytometry (FACScan; Becton Dickinson, San Jose, CA).

A second method of analyzing cell viability used the reduction of tetrazolium dye to assess mitochondrial activity as an indication of cell health. The MTT assay was performed according to manufacturer’s protocols (Cayman, Ann Arbor, MI). Briefly, cells were incubated with acrolein for 30 min. Cells were washed with PBS/10% FCS. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was added to the cells for 4 h at 37°C. The supernatant was removed and replaced with isopropanol and the optical density (540 nm) read in an automated plate reader (FLUOstar Optima).
A third method for assessing cellular viability is known as the JAM ("just another method") assay (Matzinger, 1991) and assesses the amount of intact nonfragmented DNA within cells. RBL-2H3 cells were incubated with 1 μCi/mL 3H-thymidine overnight. Cells were washed and incubated with acrolein. After incubation, cells were harvested on a semiautomated harvester and DNA deposited onto glass fiber filters, which were dried and placed into scintillation fluid and counts enumerated by scintillation spectroscopy. Total raw counts were compared between experimental conditions.

Intracellular Calcium Measurement

Intracellular calcium was determined using the FLIPR Calcium 3 Assay Kit (Molecular Devices, Sunnyvale, CA), which utilizes a calcium sensitive fluorescent dye, according to manufacturer’s instructions. Briefly, RBL-2H3 cells were grown to confluency on 96-well plates. Medium was removed and 100 μL of the FLIPR Calcium 3 assay reagent in HBSS +20 mM Heps was placed into wells and the plate incubated at 37°C for 1 h. After placing varying concentrations of acrolein, the plate was immediately placed into an automated microplate reader (FluoStar Optima) and fluorescence determined (excitation 480 nm, emission 530 nm). Results were calculated as a ratio with spontaneous fluorescence in wells containing PBS alone.

Cytokine, Chemokine, and Leukotriene Assays

RBL-2H3 cells were sensitized with DNP-IgE (200 ng/mL) for 1 h, washed, and then incubated with/without DNP and with/without acrolein and incubated for 30 min at 37°C. Cells were then washed and allowed to incubate another 24 h at 37°C. Culture supernatants were harvested and immediately frozen at −20°C until analysis. Cytokines were measured using a rat 9-plex: IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, GM-CSF, IFNγ and TNFα (Bio-Rad, Hercules, CA) and read on a Bio-Plex system (Bio-Rad) according to the manufacturer’s instructions. The assay performance reports a detection limit of <10 pg/mL with a dynamic range of 0.2–32,000 pg/mL. 14,15-leukotriene C4 (LTC4) levels were assessed using an EIA according to manufacturer’s instructions (Cayman) and optical density read (520 nm) on an automated plate reader (FLUOstar Optima).

Statistical Analyses

All analyses were performed on the means of three individual experiments, and experimental samples were performed in duplicate. Degranulation was analyzed using the Spearman correlation method and individual comparisons were made using a paired 2-sample for means t test was used, with a 95% confidence interval (p < 0.05).

RESULTS

Acrolein Induces Mast Cell Degranulation

In experiments to detect the direct effect of acrolein on mast cell degranulation, RBL-2H3 cells exposed to acrolein induced a progressive increase in the release of [H3]serotonin from 1–10,000 ppm (Fig. 1). Subsequently, we investigated the potential ability of acrolein to synergize with IgE crosslinking. To accomplish this, RBL-2H3 cells were sensitized with DNP-IgE (200 ng/mL) for 1 h, washed and then exposed to DNP with or without acrolein. Using concentrations of DNP (1 ng/mL), that induced suboptimal degranulation, there was suppression of serotonin release at acrolein concentrations of 100–10,000 ppm (Fig. 1). This suggested alternative mechanisms existed for acrolein-induced degranulation versus IgE-crosslinking.

To further explore acrolein-induced degranulation mechanisms, it has been shown previously that IgE-dependent mast cell degranulation is dependent on the influx of extracellular calcium (Lee and Oliver, 1995; Oka et al., 2002). However, we have shown previously that degranulation by a strong oxidant stimulus occurred in the absence of extracellular calcium (Collaco et al., 2006). Thus, intracellular calcium release in RBL-2H3 cells exposed to acrolein was
determined. Calcium levels increased at 0.01 ppm acrolein, and demonstrated a statistically significant increase by 10 ppm (Fig. 2). To determine if degranulation was dependent upon the influx of extracellular calcium, serotonin release was determined after extracellular calcium depletion (Fig. 3), which demonstrated no effect. Thus, we concluded that calcium stores from intracellular sources may be sufficient to induce degranulation in response to acrolein, a distinct difference from the mechanism reported previously for IgE-mediated degranulation (Lee and Oliver, 1995).

**Fig. 2.** Intracellular Ca$^{2+}$ flux. RBL-2H3 cells were treated with a calcium sensitive dye (FLPR system) and immediately read after placing concentrations of acrolein onto the wells. Values represent mean fold increases (± SEM) over cell-free wells ($n = 3$ experiments). Results show a steady rise in Ca$^{2+}$ release with increasing concentrations of acrolein. *Indicates statistically significant increase from wells with no acrolein ($p < 0.05, n = 3$). **Indicates statistical significance between 10 and 10,000 ppm acrolein ($p < 0.05, n = 3$).

**Acrolein Induces Generation of Reactive Oxygen Species**

In previous studies, the generation of intracellular ROS was associated with mast cell activation and degranulation...
Thus, we sought to investigate the mechanism of degranulation by determining the generation of intracellular ROS after acrolein exposure. Using an ROS sensitive fluorescent dye, DCFH-DA, acrolein exposed RBL-2H3 cells showed significant increases in intracellular ROS with increasing acrolein concentrations. Intracellular ROS levels increased modestly but significantly with 0.01 ppm and more substantially at 10 ppm, which was equivalent to ROS levels after exposure to 100 μM H2O2 (Fig. 4).

To investigate further, we treated cells with ROS scavengers, TMTU or NAC, which significantly reduced both ROS production and serotonin release (Fig. 5). To investigate if flavoenzymes, such as those located within NADPH oxidase or mitochondrial complex II were involved in ROS generation by acrolein, cells were treated with 100 μM DPI prior to exposure to acrolein. No change in serotonin release was observed (data not shown).

Acrolein Induces Expression of Inflammatory Cytokines

An additional indicator of mast cell activation is the secretion of inflammatory mediators, which may occur with or without degranulation (Theoharides et al., 2007). We measured the presence of cytokines in culture supernatants of RBL-2H3 cells activated with increasing concentrations of acrolein and with or without DNP-IgE/DNP. Elevated levels of cytokines (Table I) were detected only after exposure of RBL-2H3 cells to higher concentrations (10,000 ppm) of acrolein (Table I). All other concentrations of acrolein yielded undetectable or near undetectable levels. However, in contrast to degranulation experiments, there was an enhancement of cytokine release in combination with IgE crosslinking to low (1 ng/mL) and high (50 ng/mL) concentrations of DNP. Levels of LTC4 were only increased with acrolein at 1000 ppm (520 ng/mL) and 10,000 ppm (1108 ng/mL) as compared to 2380 ng/mL with DNP-IgE/DNP (50 ng/mL) alone.

Acrolein Induces Cellular Toxicity in Mast Cells

Acrolein is known to have significant cellular toxicities. To investigate the level of toxicity in our system, several methods were utilized. The MTT assay (Fig. 6) assesses the ability of cells with functional mitochondria to reduce a tetrazolium dye and is routinely used as an assessment of viability and/or cell growth. Increasing doses of acrolein incubated with RBL-2H3 cells for 30 min caused a dose-dependent loss in the ability of the cells to reduce the dye, beginning at 10 ppm and significantly at 100 ppm. This can be interpreted as loss of cellular viability and/or a loss in mitochondrial function.

PI stains the DNA of cells whose membrane integrity is compromised. To assess membrane integrity, RBL-2H3 cells were treated with acrolein for 30 min, stained with PI and analyzed by flow cytometry (Fig. 7). At 10 ppm acrolein, few cells stained with PI, but at doses of 100 ppm and greater, increasing percentages of cells demonstrated positive staining. These data imply a significant loss of membrane integrity at 100 ppm acrolein.

The JAM assay measures the loss of prelabeled DNA from cells due to the fragmentation of DNA from apoptotic cells and is considered a sensitive measure of apoptotic cell death (Matzinger, 1991). RBL-2H3 cells treated with acrolein for 30 min demonstrated a modest loss (20%) of DNA at acrolein concentrations of 1–100 ppm, while higher doses showed a progressive increase in loss up to 80% at 10,000 ppm (Fig. 8). These experiments suggested that DNA fragmentation may occur at low levels (1 ppm) but

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<th>Cytokine</th>
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*Results are expressed as pg/mL culture supernatant.

Fig. 6. Assessment of cellular viability–tetrazolium dye reduction. MTT assay assessing cellular viability in RBL-2H3 cells treated with increasing concentrations of acrolein for 30 min. Mitochondrial viability decreases substantially at 100 ppm. Values represent means (±SEM). *Indicates statistical difference from previous lower concentration (p < 0.05, n = 3).
that substantial cell death and DNA fragmentation does not occur until much higher levels (>100 ppm). Together these data indicate that acrolein is toxic to mast cells beginning at concentrations as low as 1 ppm, but that significant cell death/mitochondrial dysfunction, increased membrane permeability and loss of DNA occurs at concentrations of 100–1000 ppm.

**DISCUSSION**

Acrolein is a major component of urban air pollution. It is released in emissions and effluents from manufacturing, combustion processes (including tobacco smoking and combustion of petrochemical fuels), from direct application to water and waste water as a slimicide and aquatic herbicide, as a photo-oxidation product of various hydrocarbon pollutants found in air (including propylene and 1,3-butadiene), and from land disposal of organic waste materials (Howard, 89 A.D.; Ghilarducci and Tjeerdema, 1995; Shaughnessy et al., 2001).

**Fig. 7.** Assessment of membrane integrity-propidium iodide staining. Flow cytometry of RBL-2H3 cells exposed to increasing doses of acrolein for 30 min and stained with propidium iodide by flow cytometry. A dramatic drop in cell viability occurs at 100 ppm acrolein exposure. Values represent means (±SEM). **'** indicates statistical difference from negative control ($p < 0.05, n = 3$). 2 indicates statistical difference from previous concentration ($p < 0.05, n = 3$).

**Fig. 8.** Loss of cellular DNA-JAM assay. RBL-2H3 cells were pulsed with $[H^3]$ thymidine and treated with increasing doses of acrolein for 30 min. Cell growth rates were measured via retention of incorporated $[H^3]$ thymidine. A statistically significant drop in DNA fragmentation is shown at 1000 ppm acrolein exposure. Values represent means (±SEM). **'** indicates statistical difference between indicated concentration and negative control ($p < 0.05, n = 3$).
Epidemiologic studies have shown the physiologically relevant range of acrolein exposure is between 0.5 and 50 ppm. The average daily exposure is estimated in the range of 0.5–1.5 ppm (Caldwell et al., 1998; Woodruff et al., 1998; Tam and Neumann, 2004). The results of our experiments show a bimodal response of mast cells to acrolein exposure. High levels of exposure (>100 ppm) lead to cellular toxicity, high levels of ROS and release of mast cell inflammatory mediators, while lower levels (1–10 ppm), resulted in no toxicity nor leukotriene or cytokine release, but substantial ROS generation and degranulation. While the lowest concentrations of acrolein (<0.5 ppm) had little effect on mast cell degranulation, there was evidence for early expression of ROS and calcium release beginning at 0.01 ppm, thus, heralding the initial activation of mast cell proinflammatory pathways.

Our data demonstrating cytokine and leukotriene release at the highest levels of acrolein exposure, may be relevant in very high exposures such as might occur from direct exposure to tobacco smoke, wood fires, or acrolein processing (Ghilarducci and Tjeerdeda, 1995; Shaughnessy et al., 2001). In this situation, it is likely that release of preformed cytokines resulted from the loss of cellular membrane integrity rather than active secretory processes. The toxicity we observed may be similar to that seen in previous studies (Rahman and MacNee, 2000; Ranganna et al., 2002), where acrolein was shown to induce toxicity through the depletion of GSH and activation of MAP kinases leading to cellular growth cycle arrest and cell necrosis. At these levels, there is likely formation of DNA and protein adducts, resulting in mutation and the inability of repair mechanisms to recover damaged DNA (Kozekov et al., 2010) followed by apoptosis and DNA fragmentation. Intermediate levels of acrolein showed no evidence of cell necrosis, but still inhibited cell growth, as seen in previous studies (Kehrer and Biswal, 2000; Rahman and MacNee, 2000; Kozekov et al., 2010).

Acrolein toxicity has been linked to two major pathways. It tends to form adducts with the thiol groups of glutathione (GSH), thus depleting cellular GSH and causing antioxidant/oxidant imbalance. GSH depletion leads to activation of apoptosis, most likely through an alteration of the activity of NF-κB (Kehrer and Biswal, 2000). Kehrer and Biswal further argued that oxidative imbalance is a result of changes in GSH status rather than the formation of oxidation products themselves. Exogenous antioxidants likely alter cellular states not through blockade of ROS but through attenuation of GSH imbalance. Indirect depletion of GSH could also result from ROS generated by damage to the mitochondrial electron transport chain (Luo and Shi, 2005).

A reduction in the efficacy of superoxide dismutase (SOD) seems also to play a major role in oxidant imbalance (Cinci et al., 2010). The transcription factor Nrf2, which is responsible for SOD and GSH generation might be bound or damaged by acrolein, leading to reduction of the antioxidant generation pathway. The importance of Nrf2 in mast cell inflammatory processes was shown by Rangasamy et al. (2005).

Mast cells degranulate, in part, due to a sudden shift in cytosolic calcium levels (Strider et al., 2011), as is seen in IgE crosslinking. Unlike IgE crosslinking, which induces influx of extracellular calcium, calcium flux induced by acrolein was more likely because of release of intracellular stores of calcium. Although a precise mechanism has not been determined, acrolein may directly or indirectly induce calcium influx via activation of PKC/JNK (Misonou et al., 2005) and/or NF-κB (Woods et al., 1999).

Taken together, those previous studies corroborate the combined effects we have shown in our experiments. Acrolein exposure leads to both mast cell toxicity at higher concentrations and mast cell activation of intracellular calcium-dependent degranulation pathways resulting most likely from oxidant imbalance. Mast cell derived histamine, leukotrienes, and cytokines are key components of allergic inflammation and asthma (Holgate, 2000; Metz et al., 2007), and thus, our results support the implication of acrolein in the promotion asthma and allergic disease.

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