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Effects of intratracheal exposure of 2-chloroethyl ethyl sulfide (CEES) on the activation of CCAAT-enhancer-binding protein (C/EBP) and its protection by antioxidant liposome

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Abstract

Exposure of 2-chloroethyl ethyl sulfide (CEES) to guinea pigs causes lung injury by infiltration of neutrophils in interstitial lung spaces. A unique MAPK-regulated transcription factor, C/EBP (CCAAT-enhancer-binding protein), regulates the expression of intracellular adhesion molecule-1 (ICAM-1), involved in recruiting neutrophils in lung. The present study was to determine if CEES exposure causes activation of C/EBP, in particular the predominant β -isoform and if so whether it can be prevented by intratracheal delivery of an antioxidant liposome containing N-acetyl cysteine and tocopherols. Lung injury was developed in guinea pigs by intratracheal exposure of CEES (0.5 mg/kg). The antioxidant liposome was given intratracheally 5 min after CEES exposure, and the animals were sacrificed after 30 days. CEES exposure caused a 2.3-fold increase in the activation of C/EBP accompanied with a 45% and 121% increase in the protein level of C/EBP β and ICAM-1, respectively, and this effect was counteracted by the antioxidant liposome.

Keywords

antioxidant liposome; C/EBP (CCAAT-enhancer-binding protein); ICAM-1; 2-chloroethyl ethyl sulfide (CEES); lung injury

1 INTRODUCTION

The mustard gas, an alkylating agent, is an extensively used chemical warfare agent. Upon exposure, it is known to exert local actions on eyes, skin, and respiratory tissue followed by impairment of nervous, cardiac, and digestive systems in human and laboratory animals.^[1] We reported earlier that intratracheal instillation of 2-chloroethyl ethyl sulfide (CEES), a sulfur mustard analog, to guinea pigs, caused an increase in the expression of an inflammatory cytokine, tumor necrosis factor α (TNF α) in the lung.^[2] TNF α elevations resulted in the activation of acid and neutral sphingomyelinases and production of excessive ceramides, a second messenger involved in programmed cell death (apoptosis).^[2] In addition, intratracheal exposure to CEES led to an immediate but transient activation of NF-

κ B, which is regarded as an anti-apoptotic signaling molecule. The abrupt disappearance of NF- κ B, however, resulted in the activation of several caspases, leading to apoptosis.^[2] A follow-up study further indicated that CEES-induced activation of TNF α was associated with activation of MAP kinases in lung.^[3] CEES exposure was earlier shown to cause lung injury in guinea pigs associated with infiltration of neutrophils in the interstitial lung spaces.^[4]

It is known that neutrophil infiltration is mediated by intracellular adhesion molecule-1 (ICAM-1), a member of the immunoglobulin-like gene superfamily,^[5-8] and ICAM is directly activated by the transcription factor, CCAAT-enhancer-binding protein (C/EBP).^[9-11] Furthermore, MAPKs are activators of C/EBP.^[12] Therefore, it is hypothesized that CEES exposure activates C/EBP in lung. We reported earlier that CEES-induced lung injury is counteracted by intratracheal administration of an antioxidant liposome immediately after CEES exposure.^[13] The purpose of the present study was to investigate whether intratracheal exposure of guinea pigs to CEES causes activation of C/EBP and whether it can be counteracted by antioxidant liposome therapy.

C/EBP is a family of proteins representing a basic leucine zipper transcription factors. There are six known isoforms of C/EBP namely α , β , γ , δ , ϵ , and ζ . All isoforms exhibit similar DNA binding specificities and contain a leucine zipper dimerization region. According to our present knowledge, C/EBP β is the most prevalent form of C/EBP transcription factor in the lung.^[9] Therefore, we focused our study on C/EBP β only.

2 MATERIALS AND METHODS

2.1 Animals

Male guinea pigs (Hartley strain, 5 weeks old) were obtained from Charles River (Wilmington, MA). Animals were infused intratracheally with a single dose (0.5 mg/kg) of CEES (Sigma Chemicals, MO) in ethanol (infusion volume was 100 μ L).^[4] Control animals received vehicle only.

2.2 Antioxidant liposome

The antioxidant liposome was prepared as described earlier.^[13] The blank liposome (LIP-1) contained only soybean phospholipids, cholesterol, and phosphatidic acid in a mole fraction ratio of 71:28:0.67, respectively. The antioxidant liposome fraction (LIP-2) contained soybean phospholipids, cholesterol, and phosphatidic acid (mole fraction ratio of 55:22:0.6) along with 11 mM α -tocopherol, 11 mM γ -tocopherol, 5 mM δ -tocopherol, and 75 mM NAC.

2.3 Experimental protocol

Liposomes were given intratracheally 5 min after CEES exposure. Fifteen animals were divided into three groups. Animals of group 1 served as controls and received LIP-1 (200 μ L) after 5 min of vehicle exposure (100 μ L of ethanol). Animals of group 2 and group 3 received CEES (0.5 mg/kg body wt.), followed by either LIP-1 (200 μ L) or LIP-2 (200 μ L), respectively after 5 min of CEES exposure. All animals were sacrificed after 30 days of

CEES exposure. After sacrificing the animals, lungs were perfused with ice-cold 2.6 mM phosphate-buffered saline containing 5.6 mM glucose, removed from the chest cavity and immediately flash frozen in liquid nitrogen and stored at -80°C for further experiment. A section of the lung was fixed in buffered formalin for morphological assessment.

2.3 Morphological assessment of lung injury

It was carried out as described earlier.^[13] Briefly, a portion of lung tissue was fixed overnight in neutral buffered formalin (10%), pH 7.2, and embedded in paraffin for light microscopy. Lung sections were obtained for histological examination by staining with (a) hematoxylin and eosin and (b) trichrome.

2.4 Biochemical analysis

2.4.1 Preparation of lung extract—Lung tissues were homogenized in five volumes of ice cold lysis buffer (BioSource, Camarillo, CA) containing protease inhibitor cocktail (0.01%; Sigma Chemicals). Proteins were measured by Bio-Rad protein assay (Bio-Rad, Hercules, CA).

2.4.2 Determination of C/EBP β expression—We adopted two different approaches for monitoring C/EBP β expression: TransAM C/EBP α/β ELISA kit (Active Motif) to look for C/EBP β activation and Western blot analysis for C/EBP β protein expression.

The first approach involved plating the tissue extract in triplicate in a 96 well ELISA plate which had an immobilized oligonucleotide that contained C/EBP consensus binding site (5'-GCAAT-3'). C/EBP β present in the tissue extract specifically bound to this consensus sequence and then the primary antibody used in the kit recognized an accessible epitope on C/EBP β protein upon DNA binding.

For Western blot analysis, and 50 μg protein extract was separated by 12% SDS-PAGE. Proteins were transferred electrophoretically onto PVDF membranes (Immobilon-P Millipore, Bedford, MA). The membrane was immunoblotted with C/EBP β antibody (sc-150; Santa Cruz Biotechnology) and horseradish peroxidase-conjugated anti-mouse, anti-goat, and anti-rabbit secondary antibodies. Binding of antibodies to the blots was detected with an ECL-detection system (Perkin Elmer, Boston, MA). Stripped blots were re-probed with β -actin specific polyclonal antibodies (Santa Cruz Biotechnology Inc.) to enable normalization of signals between samples. Band intensities were analyzed using Bio-Rad Gel Doc (Bio-Rad).

2.5 Statistical analysis

Data are expressed as mean $\pm$ standard error (SE). Statistical comparison between groups was done using Turkey test after one-way analysis of variance, using GraphPad prism Version 5.0 (GraphPad Software, San Diego, CA). A *P* value of less than 0.05 was considered statistically significant.

3 RESULTS

3.1 Attenuation of CEES-induced morphological changes by antioxidant liposome

The intratracheal infusion of CEES to guinea pigs in presence of LIP-1 caused an immediate and severe injury to lung epithelium (Figure 1). CEES-induced changes in the lung included alveolar hemorrhage, fibrin and collagen deposition, influx of neutrophils, eosinophils, and parenchymal collapse as seen in human pulmonary fibrosis (bottom row, Figure 1). Furthermore, CEES exposure caused intraepithelial, intramuscular, and interstitial aggregation of red blood cells in the bronchi, alveolar space, arterioles, and veins. These morphological changes were counteracted by intratracheal infusion of the antioxidant liposome (LIP-2) given after 5 min of CEES exposure (Figure 1). Since LIP-1 was shown earlier to have no effect on CEES-induced lung injury,^[7] we did not include a group with CEES only.

3.2 C/EBP β activation by transAM C/EBP α/β ELISA kit

Figure 2 shows data on the therapeutic effects of the antioxidant liposome (LIP-2) in counteracting CEES-induced activation of C/EBP β in lung. CEES exposure caused a 3.33-fold increase in the activation of C/EBP β as evident by the observed OD values of the control group (0.252 ± 0.005 , $n = 5$) and the experimental group (0.841 ± 0.003 , $n = 5$). LIP-2 treatment counteracted the CEES-induced activation of C/EBP β by almost 50% [OD = 0.463 ± 0.003 , $n = 5$].

3.3 Western blot analysis of C/EBP β protein

Western blot analysis (Figure 3) shows data on the therapeutic effects of the antioxidant liposome (LIP-2) in counteracting CEES-induced increase in the levels of C/EBP β protein in lung. CEES exposure caused a 1.53-fold increase in the protein levels of C/EBP β as evident by the observed OD values of the control group (0.32 ± 0.01 , $n = 5$) and the experimental group (0.49 ± 0.01 , $n = 5$). LIP-2 treatment counteracted the CEES-induced increase in the levels of C/EBP β protein by almost 100% [OD = 0.32 ± 0.01 , $n = 5$].

3.4 Western blot analysis of ICAM-1

We also looked into the downstream target of C/EBP β like ICAM-1 which is involved in the neutrophil accumulation in the lung as a result of CEES exposure. Western blot analysis for ICAM-1 (ab2213; Abcam) also showed a similar pattern (Figure 4). CEES exposure caused a 2.1-fold increase in the protein levels of C/EBP β as evident by the observed OD values of the control group (0.93 ± 0.05 , $n = 5$) and the experimental group (2.0 ± 0.01 , $n = 5$). LIP-2 treatment counteracted the CEES-induced increase in the levels of C/EBP β protein by almost 60% [OD = 0.85 ± 0.01 , $n = 5$].

4 DISCUSSION

The mechanisms by which CEES exposure causes pulmonary injury/fibrosis are complex and not fully understood. We previously reported that CEES-induced lung fibrosis in guinea pig lung is associated with the activation of TNF α , MAPK signaling, and AP-1 transcription factor.^[3] Furthermore, we also reported that antioxidant liposomes (LIP-2) treatment

significantly blocked the CEES-induced activation of IL-6, IL-1 β , and SAF-1/MAZ transcription factors in guinea pig lung.^[14]

protein/CCAAT-enhancer binding protein (EBP- β) is a key intracellular regulator of inflammatory signaling pathway by modulating proinflammatory cytokines such as TNF α , IL-1, but its role in the CEES-induced pulmonary fibrosis has not been addressed earlier. This study is the first to provide evidence that C/EBP β and its downstream signaling molecule ICAM-1 in the lung mediates pulmonary inflammatory responses to CEES exposure. Since C/EBP β activity is controlled by phosphorylation,^[15] it is suggested that the activation of C/EBP β by CEES exposure is mediated by its phosphorylation status. We are currently investigating it.

As shown in Figure 1, CEES exposure caused lung injury and fibrosis associated with alveolar hemorrhage, fibrin and collagen deposition, influx of neutrophils, eosinophils, and parenchymal collapse. However, antioxidant liposome therapy counteracted these CEES-induced inflammatory response by controlling the expression of C/EBP β and its downstream signaling pathway in the airway epithelium. Thus, antioxidant liposomes containing both NAC and vitamin E (LIP-2) are effective antidote against CEES-induced pulmonary fibrosis and might offer a potential therapeutic strategy against inflammatory diseases associated with activation of these bioactive molecules.

Acknowledgments

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FIGURE 1.

Effects of antioxidant liposome treatment on CEES-induced alteration in lung morphology. Lungs were obtained after 30 days of single intratracheal instillation of either vehicle + LIP-1 or CEES (0.5 mg/kg b. wt.) + LIP 1 or CEES + LIP 2. Lung sections were obtained for histological examination by staining with H&E (top row, $\times 40$ magnification) and trichrome (bottom row, $\times 40$ magnification). In each plate, left shows blood vessels, middle shows bronchiole, and right shows alveoli.

FIGURE 2.

Protection of CEES-induced activation of C/EBP β in lung by antioxidant liposome (LIP-2) as evident by ELISA assay on C/EBP α activation. *Statistically significant ($P < 0.05$) against vehicle + LIP-1 treatment.

FIGURE 3.

Protection of CEES-induced increase in the levels of C/EBP β protein in lung by antioxidant liposome (LIP-2) as evident by Western blot analysis of the C/EBP β protein. The graph represents normalized value of C/EBP β protein against actin. *Statistically significant ($P < 0.05$) against vehicle + LIP-1 treatment.

FIGURE 4. Protection of CEES-induced increase in the protein levels of ICAM-1 in lung by antioxidant liposome (LIP-2) as evident by Western blot analysis. The graph represents normalized value of ICAM-1 against actin. *Statistically significant ($P < 0.05$) against vehicle + LIP-1 treatment.