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Research Article

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Clara cell protein mediates secretion of proteins, IL-8 and IL-6 in human airway epithelial cell line Calu-3 exposed to hyperoxia

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ABSTRACT

Clara cell 10-kD protein (CC10) is a multifunctional protein with anti-inflammatory and immunomodulatory effects. In this study we evaluated the dosage effects of recombinant human CC10 protein (rhCC10) on modulation of protein secretion in human airway epithelial cell line Calu-3. Our methodology involved testing the effects of various concentration of rhCC10 (0.5, 1.5 and 5 mg/ml) on Calu-3 cells grown at an air-liquid interface under hyperoxia insult for 24 and 48 hr. The total protein, interleukin-8 (IL8) and interleukin-6 (IL6) were measured in Calu-3 apical surface secretions by collecting apical surface fluid washings (ASF) with sterile water. Our results showed that 5 mg/ml of CC10 treatment exhibited a significant increase in total protein secretion after 24 and 48 hr under both normoxic and hyperoxic conditions. When Calu-3 cells were treated with CC10 basolaterally, a decreasing trend in total protein was observed in ASF washings indicating apical and basolateral treatment with rhCC10 exert opposing effects in protein secretory pattern. We next examined the levels of major inflammatory mediators IL-8 and IL-6 in Calu-3 protein secretions. Our results demonstrated a significant decrease in IL-8 under hyperoxia after 48 hr of CC10 treatment, whereas a steady increase in IL-6 under all conditions. These in vitro studies suggest that CC10 modulate protein secretion and inflammatory mediators in airway epithelial cells and that IL-8 and IL-6 are significantly modulated under hyperoxic insult.

Key words : Calu-3 cells, CC10, IL-8, IL-6, Hyperoxia, Inflammation.

INTRODUCTION

Clara cell secretory protein (CCSP) is the most abundant secreted protein within airways of the lung. Despite extensive characterization of the structure of CC10, there is hardly information on its physiological role in the lung. Clara cells are increasingly recognized as major respiratory tract protectors. They serve as stem cells in bronchial epithelial repair, have high xenobiotic transformation capacity, and, through the Clara cell 10-kD protein, counter regulate inflammation [1,2].

Despite extensive morphologic studies, the role of CC10 protein secreted by Clara cells in lungs remains elusive. Not even a single definitive and exclusive, physiologic role has been ascribed to these cells. The best evidence for a function for these cells points to a role in repair of bronchial epithelium. In this study we tested the effect of CC10 in airway epithelial cell line Calu-3 under hyperoxic insult. Using Calu-3 cells, we have previously developed an *in vitro* cell culture model to understand the mechanism of hyperoxia induced airway epithelial cellular injury and also to evaluate the protective role of pharmacological compounds during such hyperoxic insult [3,4].

The regulatory mechanisms of airway fluid secretion are difficult to study *in vivo*, because of its small volume. Several model systems have previously been used to functionally evaluate fluid secretions and bioelectric properties of airway epithelia [5-8]. In critically ill patients with lung disease, the respiratory epithelium is directly exposed to hyperoxia, which can cause profound cellular injury by several mechanisms such as free radical-induced cellular damages, decreased cellular proliferation, necrosis etc [9-12]. To date, *in vitro* models of hyperoxia-induced airway cell injury have employed methods other than direct exposure of cells to hyperoxic air. In the present study we tested the protein and inflammatory mediators (IL-8 & IL-6) modulated by CC10 on Calu-3 cells grown at an air-liquid interface culture by directly exposing cells to hyperoxic air.

The role of CC10 on Calu-3 cells exposed to normoxic and hyperoxic conditions will be tested by growing Calu-3 cells at an air-liquid interface. Our hypothesis is that CC10 should provide protection to the Calu-3 under hyperoxic insult by modulating inflammatory mediators such as IL-8 and IL-6. We have chosen Calu-3 cells, a well-characterized airway cell line that forms high resistance monolayers when grown on permeable supports (transwell polycarbonate porous inserts), which allows for culture at an air-liquid interface for 23 days (13,14). Under these conditions Calu-3 cells form a well-differentiated tight monolayer which generates significant TER, a characteristic of bronchiole epithelium *in vivo* (13,14). At air-liquid interface culture, Calu-3 cells develop a TER value >300 ohm.cm^2 in about 11 days, indicating formation of tight junctions in monolayers (2,3,4,13,14). Moreover, we have earlier demonstrated that the Calu-3 monolayer integrity and cellular viability were well protected during pharmacological manipulations at an air-liquid interface culture (2,3,4).

EXPERIMENTAL SECTION

At an air-liquid interface culture, Calu-3 monolayers were grown by exposing apical surface to air and cells were fed basolateraly with cell culture medium. Calu-3 monolayers will be apically exposed to normoxic ($FiCO_2$ = 5 %, balance air) or hyperoxic (FiO_2 = 95 %, balance CO_2) gas using modular incubator chamber (MIC-101, billups-rothenberg,Inc, del mar,CA) incubated at 37^{0}C. We believe this model utilizing Calu-3 grown at air-liquid interface simulates the mechanisms of lung injury recognized in clinical practice, similar in dose ratio to intra-tracheal applications in animal models (3,4).

Calu-3 cell culture

Calu-3 cells were cultured at 37° C and 5 % CO_2 in 1:1 mixture of Dulbecco's modified eagle's medium/Ham's F-12 (DMEM/F12) that was supplemented with 15 % fetal calf serum (FCS), 500 U/ml penicillin and 50 µg/ml streptomycin. Cells were maintained in 75-cm^2 tissue culture flasks and split when 80 to 90% of confluency was reached. For growing Calu-3 in transwell inserts (0.4 µm pore size, 12 mm diameter, clear polyester membrane), the cells were plated at 2 x10^6 cells/cm^2 onto transwell inserts that had been precoated with human placental collagen. The medium was added to both the apical and basolateral compartments. An air-liquid interface culture was initiated by removing medium from the apical compartment on the second day after plating cells and the apical compartment containing cells was exposed to air. The medium (0.5ml) in the basolateral compartment was changed every 2-3 days. After approximately 11 days of culturing at air-liquid interface, the TER of the monolayers was measured by adding medium (0.5ml) to the apical side and then using chopstick electrodes and an epithelial volt-ohm meter (World Precision Instruments, Sarasota, FL). Confluent monolayers with a TER value of greater than 300 ohm·cm^2 were apically treated with rhCC10 protein by adding CC10 on top of the Calu-3 monolayers and air-liquid interface culture continued. Sham treated monolayers were used as controls. All cell culture reagents were purchased from Invitrogen Corporation (Carlsbad, CA), with the exception of human placental collagen (Cohesion Technologies, Inc., Palo Alto, CA). Polyester membrane transwell-clear inserts for air-liquid interface culture was obtained from Fisher Scientific.

Culturing Calu-3 cells under normoxic and hyperoxic conditions

Calu-3 cells were exposed at normoxic ($FiCO_2$ = 5 %, balance air) or hyperoxic (FiO_2 = 95 %, balance CO_2) conditions using a modular incubator chamber (MIC-101, Billups-rothenberg, Inc, delmar, CA) at 37°C (3,4). For obtaining normoxic conditions, a transwell plate with cells and lid opened were kept inside MIC chamber, both culture plate and MIC chamber were maintained opened in CO_2 incubator (Forma Scientific, Mariotta, OH) for 30 minutes as shown in opened position of chamber. The lid of transwell tissue culture plate was also kept opened in the same way as MIC chamber. After 30 minutes of exposure, the lid was immediately placed on top of the transwell plate, and the upper and lower chambers of MIC-101 chamber were tightly closed. For obtaining hyperoxic conditions, a transwell plate with cells and lid on top was kept inside the MIC-101 chamber, lids were tightly closed

and the chamber was purged with FiO_2 = 95 %, balance CO_2 (Air gas, Radnor, PA) by opening both inlet and outlet ports. The purging of hyperoxic gas was performed at a flow rate of 20 liters/min for 3 minutes, after which the gas source was disconnected, and the chamber was sealed by closing plastic clamps. In the hyperoxic condition, the presence of a constant oxygen environment inside the experimental chambers was monitored by means of a MiniOX (oxymeter) maintained inside the chamber during the study. Both normoxic and hyperoxic experimental chambers were kept at 37°C within CO_2 incubator. The chambers were humidified by placing a petri-dish containing 20ml of sterile water inside. For both conditions, the gas in the chamber was changed every 24 hr by opening and closing the chambers.

Calu-3 cell treatment with CC10 and collection of ASF:
The effect of CC10 under hyperoxic stress will be tested for 48 hrs using 4 different concentrations of rhCC10 protein (0, 0.5, 1.5 & 5.0 mg/ml), by adding protein solution to apical surface of monolayers. Recombinant human CC10 protein was obtained as gift from Dr.Aprile L.Pilon. After treating Calu-3 monolayers with CC10, ASF was collected by washing the apical surface three times with 60 µl of sterile water and combining the washes (15,16,17). Apical surface washings in 180 µl of water were centrifuged for 15 minutes at 13,000g at 4° C and the supernatant is referred to as the ASF washing. ASF washings were assayed at the time of collection or stored in aliquots (-20° C) until used in assays.

Measurement of total proteins, IL-8 and IL-6
The secretions of proteins and inflammatory mediator's in Calu-3 cells were monitored by measuring the total protein concentration in apical surface washings (ASF) washings. The inflammatory mediators IL-8 and IL-6 levels were measured using quantitative immunossay (R & D Systems Inc., Minneapolis, MN). Data analyses will be conducted to differentiate CC10 treated and non-treated Calu-3 cells under both normoxic and hyperoxic conditions.

RESULTS

At air-liquid interface culture, analysis of Calu-3 ASF washings provides information on protein secretory activities of airway epithelial cells including inflammatory mediators. The effect of CC10 on Calu-3 protein secretory activity was tested for 24 and 48 hrs under both normoxic and hyperoxic conditions. Our results showed no difference in the levels of total protein secretion between normoxic and hyperoxic conditions in the absence of CC10. At 0.5 and 1.5 mg/ml of CC10 concentration, no change in protein secretion was observed (Fig 1). However, at 5 mg/ml of CC10 concentration, a significant increase in total protein secretion was seen after 24 and 48 hr under both normoxic and hyperoxic conditions when compared with 0.5 and 1.5 mg/ml of CC10 treatment. Interestingly, no difference in protein concentration was found between normoxic and hyperoxic condition at 5 mg/ml of CC10 treatment.

As we observed an increase in total protein after 48 hr of CC10 treatment under hyperoxia, we next tested the changes in total protein upto 72hr of hyperoxia treatment, when CC10 was treated in apical surface. This study demonstrated a steady increase in total protein in ASF washings (Fig2). Interestingly, when Calu-3 cells were treated basolaterally with CC10, a decreasing trend in total protein was observed in ASF washings, indicating apical and basolateral CC10 treatment exert opposing effects in protein secretory pattern (Fig3).

Considering the immunomodulatory and anti-inflammatory effects of CC10, we examined the changes in major inflammatory mediators IL-8 and IL-6 in Calu-3 protein secretions. Our studies showed a dose dependent increase in IL-8 secretion under normoxic conditions up to 48 hr of CC10 treatment, but 5mg/ml did not alter IL-8 secretion (Fig4). After 48 hr, all concentrations of CC10 resulted in a significant decrease in IL-8 under hyperoxic condition compared to normoxic condition. After 24 and 48 hr, at all concentrations of CC10 a dose dependent increase in IL-6 secretion was seen under normoxic and hyperoxic conditions (Fig5). Therefore, we observed a significant decrease in IL-8 under hyperoxia after 48 hr of CC10 treatment, whereas a steady increase in IL-6 under all conditions.

DISCUSSION

In the present study we used Calu-3 cells, a well-characterized airway cell line that forms high resistance monolayers at an air-liquid interface, which enabled us to evaluate the effect of CC10 treated apically and basolaterally. In addition to the advantages of air-liquid interface culture, other aspects of Calu-3 function are similar to the *in vivo* condition and primary airway culture models. For example, we have previously observed the

presence of basal level fluid and protein secretions in Calu-3 apical surface at an air-liquid interface (18-22), similar to protein secretions of airway primary cultures (6,7,8).

The Calu-3 cell line has previously been employed as a model to screen possible drug candidates and formulations to be delivered to the respiratory epithelium [11-14]. The pharmacological effects of synthetic drugs and steroids have previously been tested in asthma and other airway diseases (23,24). Calu-3 cells grown at air-liquid interface simulates pulmonary airway epithelial cells *in vivo*, wherein cells are under constant exposure to environmental air, and in previous studies we demonstrated that hyperoxic conditions induced cellular injuries in Calu-3 grown at air-liquid interface. Taking advantage of Calu-3 cells growing at an air-liquid interface [3,4,15,18,21,22], we were able to evaluate the protein modulatory effects of CC10. Such a study is highly beneficial in understanding the physiological and pharmacological effects of CC10 when cells are grown in direct contact with air.

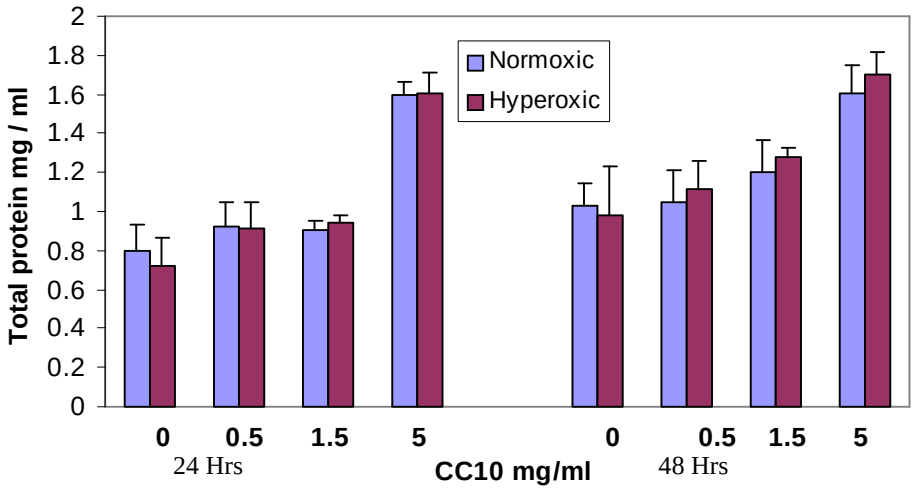

Fig 1: Total protein concentration in Apical Surface Fluid (ASF) washings was measured by DC protein assay. total protein concentration (n=4). The results were compared between normoxic and hyperoxic conditions at 24 and 48 hr of CC10 treatment

We have earlier demonstrated that Calu-3 cells secrete protein components associated with inflammation and innate immunity and identified pro-inflammatory cytokine IL-8 as a major cytokine mediator secreted by Calu-3 cells [3,21]. In the present study we evaluated the role rhCC10 in modulating total protein secretions as well as inflammatory mediators such as IL-8 & IL-6 secretions in airway epithelial cells under hyperoxic insult. The studies of Joshua et al (2010) provided evidence that clara cells can attenuate inflammation through regulation of macrophage behavior, and suggest that epithelial remodeling leading to reduced Clara cell secretory function is an important factor that increases the intensity of lung inflammation in chronic lung disease (25). CC10 has earlier been shown to inhibit interferon-γ, tumor necrosis factor-α, and interleukin-1 β. Both the murine and human CC10 promoter regions contain sites where inflammatory mediators, such as tumor necrosis factor-α and interferon-α, -β and -γ, alter transcriptional activity (26,27). It is also well known that pulmonary epithelial cells can also be a source of inflammatory mediators (28-30) and IL-8 release from alveolar epithelial cells (A549) has previously been reported by several investigators (28-31).

The Calu-3 cell culture model developed in the above study will be helpful to investigate potential therapeutic benefit of CC10 protein and understanding the mechanism of regulation of inflammation by CC10. Our hypothesis is that CC10 should provide protection to the Calu-3 cell line when exposed to a hyperoxic insult. Based on our

observations, we suggest that rhC10 administration modulate protein secretions and inflammatory mediators during the clinical management of respiratory inflammation

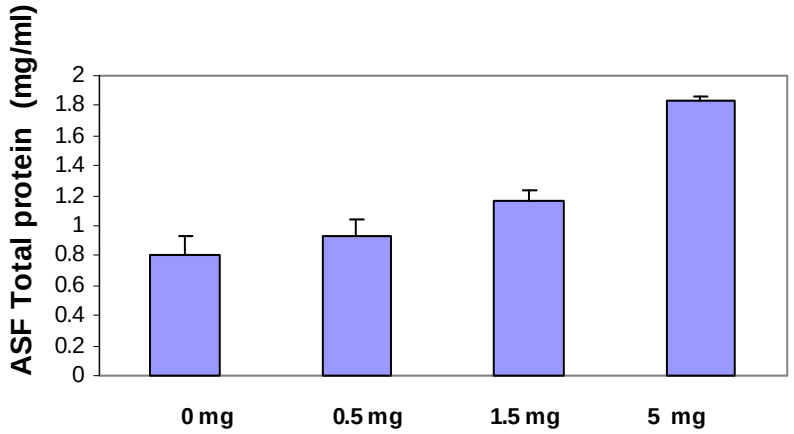

Figure 2 : Total protein concentration in apical surface fluid (ASF) washings was measured by DC protein assay. Different concentration of CC10 was added to the apical surface of Calu-3 cells grown at air-liquid interface culture. The samples were tested for total protein after 72 hr of Hyperoxia exposure (n=4).

Figure 3

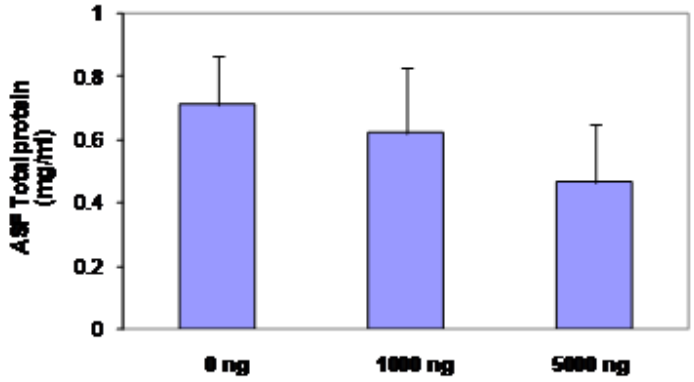

Figure 3 : Total protein concentration in apical surface fluid (ASF) washings was measured by DC protein assay. Calu-3 cells were treated with CC10 protein by adding CC10 basolaterally in the culture medium at an air-liquid interface culture and cells were exposed for Hyperoxia for 72 hrs (n=4).

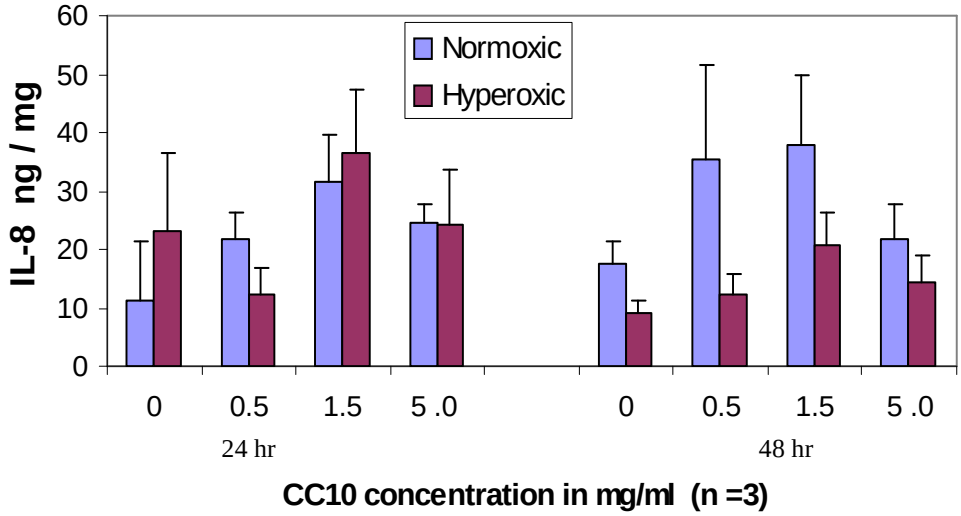

Figure 4 : Calu-3 cells were treated with CC10 under hyperoxia and normoxia for 24 and 48 hrs. The secretions of IL-8 in apical surface fluid (ASF) washings was measured using commercial kit obtained from R & D Systems Inc.,Minneapolis, MN (n=3). The IL-8 levels were measured in ng/mg of total ASF protein.

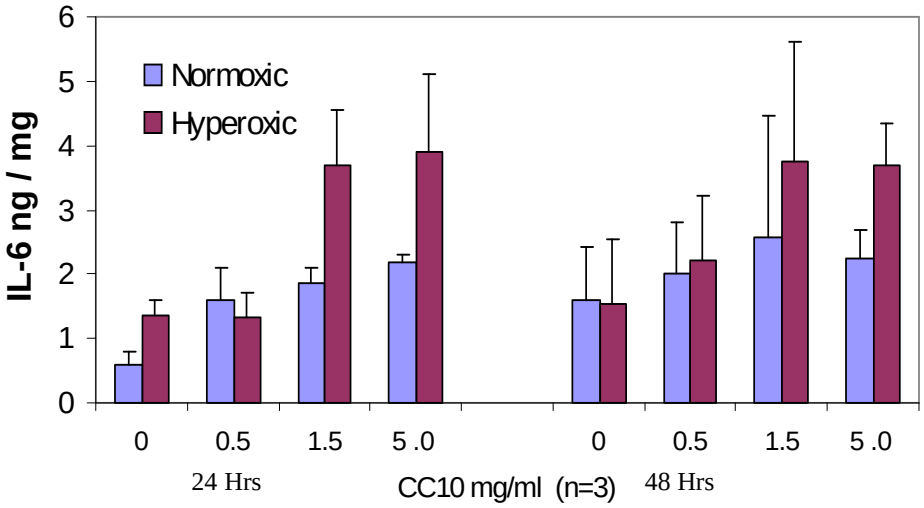

Figure 5 : Calu-3 cells were treated with CC10 under hyperoxia and normoxia for 24 and 48 hrs. The secretions of IL-6 in apical surface fluid (ASF) washings was measured using commercial kit obtained from R & D Systems Inc., Minneapolis, MN (n=3). The IL-6 levels were measured in ng/mg of total ASF protein.

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