Clara cell secretory protein deficiency increases oxidant stress response in conducting airways

GREGORY W. MANGO,1 CARL J. JOHNSTON,1,2 SUSAN D. REYNOLDS,1 JACOB N. FINKELSTEIN,1,2 CHARLES G. PLOPPER,3 AND BARRY R. STRIPP1,2

Departments of 1Environmental Medicine and 2Pediatrics, University of Rochester, School of Medicine and Dentistry, Rochester, New York 14642; and 3Department of Anatomy, Physiology, and Cell Biology, School of Veterinary Medicine, University of California, Davis, California 95616

Mango, Gregory W., Carl J. Johnston, Susan D. Reynolds, Jacob N. Finkelstein, Charles G. Plopper, and Barry R. Stripp. Clara cell secretory protein deficiency increases oxidant stress response in conducting airways. Am. J. Physiol. 275 (Lung Cell. Mol. Physiol. 19): L348–L356, 1998.—Little is known about the molecular basis for differential pulmonary oxidant sensitivity observed between genetically disparate members of the same species. We have generated mice that are deficient in Clara cell secretory protein (CCSP) and that exhibit an oxidant-sensitive phenotype. We characterized the kinetics and distribution of altered stress-response [interleukin-6 (IL-6) and metallothionein (MT)] and epithelial cell-specific [cytochrome P-450 2F2 (CYP2F2)] gene expression to further understand the cellular and molecular basis for altered oxidant sensitivity in 129 strain CCSP−/− mice. Increases in IL-6 and MT mRNA abundance were detected by 2 h of exposure to 1 part/million ozone and preceded reductions in Clara cell CYP2F2 mRNA expression. Despite being qualitatively similar, increases in IL-6 and MT mRNA expression were enhanced in CCSP−/− mice with respect to coexposed 129 strain wild-type mice. Increased MT mRNA expression, indicative of the stress response, localized to the airway epithelium, surrounding mesenchyme, and endothelium of blood vessels. These results demonstrate a protective role for Clara cells and their secretions and indicate potential genetic mechanisms that may influence susceptibility to oxidant stress.

uteroglobin; cytokines; metallothionein; ozone; lung injury

Although Clara cells have been identified and investigated in many species, their role in normal lung function remains largely speculative. Clara cells are responsible for secretion of nonmucoid substances into the airway lumen and therefore contribute to the maintenance of extracellular lining fluid (ELF) composition (11, 21, 43). One of the most abundant ELF proteins secreted by Clara cells is a 16-kDa homodimer, referred to as Clara cell 10-kDa secretory protein (CC10, CCSP, or CC16), an ancestral homolog of rabbit uteroglobin (44, 50, 52). The true in vivo function of CCSP is unknown. Insight into potential functions of CCSP has been derived from analysis of its biological and biochemical properties. These properties include the ability to bind progestins and other small lipophilic compounds (8, 36, 49, 50), inhibit secretory phospholipase A2 (31), inhibit phagocyte chemotaxis (56), and bind calcium (3).

The spatial distribution and abundance of Clara cells found within the airway epithelium differ among species. However, Clara cells of all species are considered to be one of the more oxidant-resistant airway cell types. In the normal rodent lung, Clara cells actively participate in regeneration of the epithelium after oxidant-induced damage to ciliated cells (14, 15, 33). The possible involvement of Clara cells and their secretions in protection from pulmonary oxidant stress has been inferred from a number of studies. In the rat lung, adaptation to chronic ozone exposure is associated with airway remodeling, characterized by increases in the number of Clara cells in distal airways (7, 38, 41, 51) and increases in the abundance of CCSP and activity of antioxidant enzymes (13, 42). These data support the hypothesis that tolerance to chronic oxidant stress may be associated with increased secretory capacity of conducting airways, arising from both increased Clara cell numbers and altered Clara cell secretion. Oxidant-induced alterations in the secretory capacity of Clara cells can also be inferred from studies investigating acute responses of conducting airways to inhaled NO2. Acute exposure of rats to NO2 is associated with loss of Clara cell secretory granules, suggesting oxidant-induced secretion (14). It remains to be determined what contribution, if any, constitutive and/or inducible Clara cell secretion makes to antioxidant capacity of the ELF and protection of airways from oxidant injury.

It is well established that genetic factors influence susceptibility to a variety of pneumotoxic agents, including ozone (2, 27–29, 45). Other studies investigating genetic susceptibility to bleomycin-induced lung fibrosis have demonstrated a positive correlation between induction of IL-6 mRNA expression and the sensitivity of mouse strains (6). Tissue injury is a potent stimulus for initiation of the stress response, otherwise known as the acute-phase response. Interleukin-6 (IL-6) is the major cytokine involved in the upregulation of acute-phase proteins in the liver, and this function allows IL-6 to serve as a long-distance messenger of focal injury (18, 47). Metallothionein (MT) is another stress-responsive gene that has been shown to be regulated in the lung in response to a variety of injury stimuli, including oxidant stress (25, 37, 46). It has been suggested that MT may function as an antioxidant (19, 46, 55) and therefore may serve an important protective function during the acute-phase response (34, 39).

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To define in vivo roles for CCSP and Clara cell secretions, we have generated a line of genetically modified mice that are homozygous for a null allele of the CCSP gene (53). We have shown that CCSP-deficient (CCSP −/−) mice are more sensitive to hyperoxia and exhibit alterations in inflammatory cytokine gene expression (23). In the present study, we investigate changes in the expression of IL-6 and MT mRNAs as biomarkers for the initial phase of the stress response associated with ozone exposure in wild-type (WT) and CCSP −/− mice. We also examined the expression of cytochrome P-450 2F2 (CYP2F2), which is only expressed at high levels within Clara cells of the murine lung (43), to assess integrity of the airway epithelium, and specifically Clara cells, after ozone exposure. We demonstrate that CCSP deficiency enhances the oxidant-induced stress response in CCSP −/− mice. The increase in stress response was characterized by a dramatic but transient increase in IL-6 mRNA abundance and a more protracted elevation in the abundance of MT mRNA. Results from in situ hybridization analysis show that the altered MT mRNA expression of CCSP −/− mice localizes to airway epithelia, surrounding mesenchyme, and the endothelial cells lining blood vessels. These results demonstrate a role for Clara cells and their secretions in protection from ozone-induced lung injury.

MATERIALS AND METHODS

Animals. WT strain 129 (Taconic, Germantown, NY) and CCSP −/− strain 129 mice were maintained as specific pathogen-free, in-house colonies and were allowed food and water ad libitum. Male mice between the ages of 2 and 5 mo were used for all experiments.

Exposures. WT and CCSP −/− mice were coexposed to ozone in Rochester chambers (30, 57) for the indicated times. Ozone was generated by exposing 100% O2 to ultraviolet light using an OREC model 03V1–0 ozone generator (Ozone Research and Equipment, Phoenix, AZ) and was diluted with filtered room air. Mice were maintained in wire mesh cages with ozone concentration measured at nose level and continuously recorded using a Dasibi 1003-AH ozone analyzer (Dasibi Environmental, Glendale, CA) calibrated against a New York State Department of Environmental Conservation ozone calibrator. Ozone concentration was maintained at 1.0 ± 0.1 part/million (ppm) for time-course studies and at either 0.5 ± 0.1, 1.0 ± 0.1, or 2.5 ± 0.1 ppm for dose-response analysis. Control mice were exposed to filtered room air in an adjacent chamber.

Tissue collection. Mice were killed immediately after removal from ozone by intraperitoneal injection of 100 mg/kg pentobarbital sodium followed by exsanguination. For time-course analysis, three to six mice were exposed for any one

Fig. 1. Results from S1 nuclease protection assays on 5 µg of total lung RNA using 32P-labeled DNA probes. A: autoradiogram of representative samples. Lanes show hours (indicated above each lane) of continuous 1.0 part/million (ppm) ozone exposure for wild-type (WT) and Clara cell secretory protein (CCSP)−/− mice. Results illustrate decreases in cytochrome P-450 2F2 (CYP2F2) mRNA and increases in metallothionein (MT) mRNA after ozone exposure. Message for the ribosomal protein L32 (L32) is shown as an internal standard. B: quantitation (n = 3 independent mice/pool) of CYP2F2 mRNA expression in WT (filled bars) and CCSP −/− (hatched bars) mice normalized to the internal standard L32. y-Axis shows relative mRNA abundance. x-Axis shows hours of continuous 1.0 ppm ozone exposure. *Significant changes with respect to corresponding WT or CCSP −/− filtered air controls (0 h). Significance was assessed at P < 0.05. C: similar to B, but quantitative changes in MT mRNA abundance are shown. †Significant changes in CCSP −/− mice with respect to coexposed WT mice at the time points indicated.
treatment, of which two mice from each group were used for both isolation of total RNA and tissue fixation. Total lung RNA was isolated from remaining mice. For RNA isolation and tissue fixation, lungs were exposed, and the left lobar bronchus was tied using suture silk. Left lobes were removed, homogenized in guanidine thiocyanate solution (10), and stored at −20°C until RNA was isolated. Tracheae were then cannulated with an Inspyte 20-gauge intravenous catheter. Right lobes were fixed by inflation with ice-cold 2% glutaraldehyde in 100 mM cacodylate buffer (pH 7.4). Lungs were inflated to capacity with fixative and then allowed to stand for 10 min at 10 cmH₂O pressure, followed by immersion in the same fixative for an additional 8 h at 4°C. Fixed lung tissue was washed in 100 mM cacodylate buffer (pH 7.4) at 4°C; lower (caudal) right lobes were removed, dehydrated, and embedded in paraffin; and 5-µm sections were prepared for in situ analyses. For dose-response studies, lungs were removed from exposed mice, and total RNA was isolated.

RNA isolation and analysis. Total lung RNA was isolated by the method of Chomczynski and Sacchi (10). Cytokine mRNA expression was determined by RNase protection assay (RPA) using the RiboQuant mCK-2 multiprobe set (PharMin- gen, San Diego, CA). Conditions used have been described previously (23). In brief, 32P-radiolabeled riboprobes were synthesized based on the protocol by Melton et al. (35), with slight modifications. Excess probe was hybridized to 5 µg of total RNA at 56°C overnight. Unbound probe and single-stranded RNA were digested using RNase A and RNase T1.

Fig. 2. Representative light micrographs showing in situ localization of CYP2F2 mRNA. Lung tissue sections from CCSP −/− mouse lung exposed to filtered air (A and B) or 4 h (C and D) of 1.0 ppm ozone. Sections were hybridized to 32P-labeled antisense CYP2F2-specific riboprobe, and hybridization was localized by autoradiography. Photomicrographs were taken using either ×10 (A and C) or ×40 (B and D) objective. White boxes indicate terminal bronchiole-alveolar duct junctions from low-magnification photomicrographs that are shown in the higher-magnification images. Silver grains localizing hybridized 32P-labeled riboprobe are shown as white grains in pseudo-dark-field images (A and C) and dark grains in bright-field images (B and D).
6% acrylamide denaturing gel, as with RPAs. Both RPA and S1 results were quantitated using a PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

In situ hybridization. In situ hybridization for CYP2F2 and MT mRNA has been described previously (22, 53). Probes were synthesized based on published methods (35). Linearized plasmids containing mouse CYP2F2 cDNA and MT (a gift from G. Andrews, Kansas University) served as templates to generate antisense riboprobes using either SP6 or T7 RNA polymerase in the presence of [33P]UTP. Five-micrometer sections from paraffin-embedded caudal right lobes (see above) were probed for mRNA localization. Tissue section preparation and hybridization conditions followed those described previously (4, 23). After hybridization, slides were RNase treated and washed. Slides were then dehydrated and dipped in Kodak NTB2 photographic emulsion (Kodak, Rochester, NY).

Statistics. Two-way ANOVA was done using SuperAnova software (Abacus Concepts, Berkeley, CA) to compare results between coexposed WT and CCSP −/− mice. Post hoc least squares analysis was used to assess significance. After two-way ANOVA, one-way ANOVA was used to independently compare results from WT and CCSP −/− mice with their corresponding filtered air-exposed controls. Post hoc Fisher’s protected least significant difference analysis was used to assess significance. For all analyses, significance was assessed at P ≤ 0.05.

RESULTS

Ozone exposure alters Clara cell gene expression. To investigate the hypothesis that CCSP deficiency alters the response of conducting airways to oxidant stress, we initially examined changes in CYP2F2 mRNA expression in ozone-exposed WT and CCSP −/− mice. Within the mouse lung, only Clara cells express high levels of CYP2F2 (43). We have previously demonstrated that CCSP deficiency is not associated with altered steady-state levels of CYP2F2 mRNA (53). Therefore, we investigated the kinetics of ozone-induced changes in CYP2F2 as a potential marker of differential ozone susceptibility. Figure 1 shows the results of S1 nuclease protection assays performed using total lung RNA. Ozone-induced reductions in CYP2F2 mRNA levels were observed in both WT and CCSP −/− mice. No significant changes in CYP2F2 mRNA levels were observed in either WT or CCSP −/− mice until 8 h of ozone exposure, at which time levels were reduced to 69% (P = 0.105) and 47% (P = 0.020) of filtered air control levels, respectively. Regional alterations in CYP2F2 mRNA abundance were characterized by in situ hybridization of lung tissue sections using an antisense riboprobe (Fig. 2). Results demonstrated that, within CCSP −/− mice, CYP2F2 mRNA was reduced in Clara cells of terminal bronchioles by 4 h of continuous exposure to 1.0 ppm ozone. These data demonstrate that, despite regional changes in CYP2F2 mRNA expression, alterations in total lung CYP2F2 mRNA abundance do not provide a sensitive measure of the early response to ozone exposure. Because the goal of the present study was to define early events associated with the differential oxidant sensitivity of CCSP −/− mice relative to strain-matched WT mice, further studies involved analysis of stress-response gene expression.

Ozone-mediated induction of MT and IL-6 mRNAs is dramatically enhanced with CCSP deficiency. Unlike CYP2F2, basal expression of MT and IL-6 mRNAs was low, allowing increases resulting from oxidant injury to be measured with greater sensitivity. Analysis of MT mRNA expression by S1 nuclease protection assay (Fig. 1) and IL-6 mRNA expression by RPA (Fig. 3) revealed similar baseline levels in filtered-air-exposed control WT and CCSP −/− mice. Both MT and IL-6 mRNAs were differentially regulated between WT and CCSP −/− mice after ozone exposure.

Fig. 3. Results from RNase protection assays on 5 µg of total lung RNA using 32P-labeled RNA probes. A: autoradiogram of representative samples. Lanes show hours (indicated above each lane) of continuous 1.0 ppm ozone exposure for WT and CCSP −/− mice. Results illustrate a transient increase in interleukin-6 (IL-6) mRNA after ozone exposure. Macrophage inhibitory factor (MIF) is included in the probe set used (see MATERIALS AND METHODS) but shows no ozone-dependent alterations in mRNA abundance. Measurements of mRNA for the ribosomal protein L32 were included as an internal standard. B: quantitation (n = 3 independent mice/point) of IL-6 mRNA expression in WT (filled bars) and CCSP −/− (hatched bars) mice, normalized to the internal standard L32. y-Axis shows relative mRNA abundance. x-Axis shows hours of continuous 1.0 ppm ozone exposure. *Significant changes with respect to corresponding WT or CCSP −/− filtered air controls (0 h). †Significant changes in CCSP −/− mice with respect to coexposed WT mice at the time points indicated. Significance was assessed at P ≤ 0.05.
Significant increases in MT mRNA abundance were observed in CCSP −/− mice beginning at 2 h of ozone exposure relative to filtered air controls (Fig. 1). Levels of MT mRNA remained elevated at ∼15-fold over corresponding filtered air controls at both 4- and 8-h time points in CCSP −/− mice. In contrast, ozone-induced changes in MT mRNA abundance in WT mice, despite showing an upward trend at 4 h, were not significantly elevated until 8 h of ozone exposure. MT mRNA abundance in 8-h ozone-exposed WT mice was comparable to that detected in CCSP −/− mice at the 4-h time point (14- vs. 14.5-fold, respectively; Fig. 1). Therefore, in ozone-exposed CCSP −/− mice, MT mRNA showed altered kinetics but similar magnitude of induction relative to similarly exposed WT mice (Fig. 1C).

Ozone-induced increases in IL-6 mRNA were transient and showed peak mRNA levels at 4 h of ozone exposure in both WT and CCSP −/− mice (Fig. 3). However, despite similarities in the kinetics of induction, ozone-induced increases in IL-6 mRNA showed quantitative differences between WT and CCSP −/− mice. IL-6 mRNA was elevated 3.9- and 14.1-fold at the 4-h ozone exposure time point in WT and CCSP −/− mice, respectively. Increases in IL-6 mRNA were transient, returning to near-control levels in both WT and CCSP −/− mice by 8 h of ozone exposure (Fig. 3). Therefore, in contrast to the pattern of differential MT expression between WT and CCSP −/− mice, ozone-elicited increases in IL-6 mRNA expression showed similar kinetics but differences in the magnitude of induction (Fig. 3B).

CCSP −/− mice show induction of MT mRNA expression within airway epithelium and adjacent tissues. Differential MT and IL-6 mRNA expression demonstrates increased ozone-induced oxidative stress associated with CCSP deficiency. These changes in MT mRNA expression were localized to determine 1) if these changes occurred in conducting airways (based on the pattern of CCSP expression in WT mice) and 2) if other cell types, in addition to those of conducting airways, exhibited ozone-induced oxidant stress in CCSP −/− mice. Consistent with results from S1 nuclease protection assays (Fig. 1), robust expression of MT mRNA was detected in lung tissue of ozone-exposed CCSP −/− mice after 4 h of ozone exposure. At this time, MT mRNA localized to epithelial cells of conducting airways, mesenchymal tissue surrounding conducting airways, and endothelial cells of blood vessels (Fig. 4, C and D). No specific hybridization signal was observed in lung tissue from filtered air-exposed mice.
CCSP−/− mice (Fig. 4, A and B) or in lung tissue from either filtered air- or 4-h ozone-exposed WT mice (data not shown).

WT mice show similar changes to CCSP−/− mice in IL-6 and MT mRNA expression at higher doses of ozone. Altered kinetics of MT mRNA expression in ozone-exposed CCSP−/− mice relative to WT mice demonstrates a shift in the time-course response toward increased sensitivity. However, because IL-6 mRNA expression differed between WT and CCSP−/− mice in magnitude but not in kinetics of the response, dose response rather than time-course analyses were required to better define the sensitivity relationship.

Figure 5 shows the results of dose-response analysis characterizing the expression of IL-6 and MT mRNA in lungs of WT and CCSP−/− mice after exposure to 0, 0.5, 1, and 2.5 ppm ozone for 4 h. MT mRNA expression in 2.5 ppm ozone-exposed WT mouse lungs was similar to that observed in the lungs of CCSP−/− mice after exposure to 1.0 ppm ozone (Fig. 5, A and C). Interestingly, MT mRNA abundance showed no further increases in CCSP−/− mice beyond the 1.0-ppm ozone dose. Similar results were obtained for altered IL-6 mRNA expression. As with altered MT mRNA, IL-6 mRNA increased in 2.5 ppm ozone-exposed WT mouse lungs to similar levels to that observed in lungs of 1.0 ppm-exposed CCSP−/− mice (Fig. 5, B and D). Again, as was the case for the MT mRNA dose response, IL-6 mRNA abundance showed no further increases in CCSP−/− mice beyond the 1.0 ppm ozone dose. These data are consistent with the conclusion from time-course analysis that ozone sensitivity is enhanced in CCSP−/− mice relative to strain-matched WT mice.

DISCUSSION

In the present study, we show that CCSP deficiency enhances the ozone-stimulated pulmonary stress response. Increases in the abundance of IL-6 and MT mRNAs served as sensitive end points, allowing early discrimination of differential ozone sensitivity associated with CCSP deficiency. Both MT and cytokines, such as IL-6, are known to be regulated by intracellular
redox potential, stress resulting from cell/tissue injury, and inflammation (1, 9, 12, 32). Expression of IL-6 and MT occurs early in the stress response, both before and during inflammatory cell recruitment (9, 22, 48). Furthermore, the inflammatory response has the potential to perpetuate cytokine and stress-response gene expression (20, 32, 34). Increases in ozone-induced stress-response gene expression in CCSP −/− mice demonstrate a defect in the pulmonary response to oxidant pollutants. This is further supported by the finding that CCSP deficiency results in a shift in the dose-response relationship toward increased sensitivity. In addition, our parallel studies demonstrate increased epithelial cell necrosis between WT and CCSP −/− mice after 8 h of continuous exposure to 1.0 ppm ozone (Plopper, unpublished observations). Based on these findings, we speculate that the increased oxygen sensitivity associated with CCSP deficiency is suggestive of a role for CCSP and/or Clara cells in pulmonary antioxidant defense.

There are no published studies describing antioxidant activities associated with CCSP. Interestingly, tolerance associated with chronic ozone exposure has been associated with elevations in the pulmonary content of CCSP and antioxidant enzymes (13, 42). Activities associated with the protein that could contribute to antioxidant defense include the ability to bind divalent cations and lipophilic compounds (3, 8, 36, 49, 50). It is equally likely that CCSP deficiency has an indirect influence on antioxidant defenses in the lung. Clara cells have been shown to lose their secretory granules after oxidant stress (14). Although the contribution that oxidant-induced Clara cell degranulation makes to protection from oxidant stress is unknown, we have previously shown that Clara cells of CCSP −/− mice lack secretory granules and possess other changes to their secretory apparatus, including reduced cytoplasmic volume of rough endoplasmic reticulum (40, 53). These ultrastructural alterations, if they translate into functional changes in Clara cell secretion and/or protein synthesis, could potentially account for the observed oxidant sensitivity of CCSP −/− mice. This would suggest an important role for Clara cells and oxidant-induced Clara cell secretion as an immediate protective response to oxidant stress.

Genetic mechanisms accounting for pollutant sensitivity, particularly the sensitivity to oxidant pollutants, are poorly defined. Considerable variability is observed between mouse strains in their sensitivity to pollutant injury and particularly oxidant stress (17, 24, 27, 58). Much of this variability has been attributed to genetic background, but the responsible genes have not been identified (2, 6, 28, 29, 45). Because the CCSP −/− mice presented herein were established in an inbred 129 genetic background, we attribute the resulting oxidant sensitivity to this single genetic alteration. However, genetic alterations, such as the introduction of a null allele of the CCSP gene, have the potential for pleiotropic effects. Further studies are needed to determine whether CCSP deficiency per se or changes in either Clara cell function or the functions of other pulmonary epithelial cells are the basis for oxidant sensitivity in CCSP −/− mice. In addition, further investigation is required to determine whether pulmonary changes associated with CCSP deficiency are in any way related to previously characterized strain differences in oxidant sensitivity.

Previously, we have shown that CCSP −/− mice are more sensitive to hyperoxia exposure and displayed altered cytokine gene expression. Possible explanations for this differential response are altered sensitivity of the lung to oxidant injury and/or altered regulation of the inflammatory response. Studies presented herein support the concept that CCSP deficiency increases the sensitivity of the lung to direct oxidant-induced stress. We conclude that the CCSP-deficient mouse model has utility to define a role of CCSP and/or Clara cells in protection from oxidant stress. However, due to differences in the sensitivity of pulmonary epithelial cells to oxidant stress and likely alteration of injury-induced inflammation, we are unable to draw conclusions regarding a direct role for CCSP in regulation of pulmonary inflammatory responses.

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