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Broeckaert, F ; Arsalane, K. ; Hermans, Cédric ; Bergamaschi, E. ; Brustolin, A ; Mutti, A ; Bernard, Alfred

ABSTRACT

Ozone in ambient air may cause various effects on human health, including decreased lung function, asthma exacerbation, and even premature mortality. These effects have been evidenced using various clinical indicators that, although sensitive, do not specifically evaluate the O(3)-increased lung epithelium permeability. In the present study, we assessed the acute effects of ambient O(3) on the pulmonary epithelium by a new approach relying on the assay in serum of the lung-specific Clara cell protein (CC16 or CC10). We applied this test to cyclists who exercised for 2 hr during episodes of photochemical smog and found that O(3) induces an early leakage of lung Clara cell protein. The protein levels increased significantly into the serum from exposure levels as low as 0.060-0.084 ppm. Our findings, confirmed in mice exposed to the current U.S. National Ambient Air Quality Standards for O(3) (0.08 ppm for 8 hr) indicate that above the present natural background levels, there is almost no safety margin for the effects of ambient O(3) on airway permeability. The assay of CC16 in the serum represents a new sensitive noninvasive test allowing the detection of early effects of ambient O(3) on the lung epithelial barrier.

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Serum Clara Cell Protein: A Sensitive Biomarker of Increased Lung Epithelium Permeability Caused by Ambient Ozone

Fabrice Broeckaert,1 Karim Arsalane,1 Cédric Hermans,1 Enrico Bergamaschi,2 Angelita Brustolin,2 Antonio Mutti,2 and Alfred Bernard1

1Unit of Industrial Toxicology and Occupational Medicine, Faculty of Medicine, Catholic University of Louvain, Brussels, Belgium; 2Laboratory of Industrial Toxicology, Medical School, University of Parma, Parma, Italy

Ozone in ambient air may cause various effects on human health, including decreased lung function, asthma exacerbation, and even premature mortality. These effects have been evidenced using various clinical indicators that, although sensitive, do not specifically evaluate the O3-increased lung epithelium permeability. In the present study, we assessed the acute effects of ambient O3 on the pulmonary epithelium by a new approach relying on the assay in serum of the lung-specific Clara cell protein (CC16 or CC10). We applied this test to cyclists who exercised for 2 hr during episodes of photochemical smog and found that O3 induces an early leakage of lung Clara cell protein. The protein levels increased significantly into the serum from exposure levels as low as 0.060–0.084 ppm. Our findings, confirmed in mice exposed to the current U.S. National Ambient Air Quality Standards for O3 (0.08 ppm for 8 hr) indicate that above the present natural background levels, there is almost no safety margin for the effects of ambient O3 on airway permeability. The assay of CC16 in the serum represents a new sensitive noninvasive test allowing the detection of early effects of ambient O3 on the lung epithelial barrier. Key words: biomarker, CC10, CC16, Clara cell protein, epithelium, lung permeability, ozone. Envir Health Perspect 108:533–537 (2000). [Online 26 April 2000] http://ehpnet1.niehs.nih.gov/docs/2000/108p533-537/broeckaert/abstract.html

Ozone, the main oxidant species of photochemical smog, can produce a variety of acute pulmonary effects, including decreme

Materials and Methods

Study on cyclists. The study was conducted in Parma, Italy, between 18 June and 31 July 1998 under varying conditions that included episodes of photochemical smog. After providing their informed consent, 24 nonsmoking cyclists (15 women and 9 men) 28.5 (SD, 3.4) years of age participated in the study. Each volunteer performed two 2-hr rides between 0200 and 0400 hr on roads and dates characterized by different levels of air pollution. The rides covered a distance between 30 and 40 km (18 and 25 miles). The speed was moderate (9–13 miles/hr) and the heart rate was relatively stable (122.3 ± 11.8 beats/min). Ozone concentration was monitored every 10 min by six stations of the local monitoring network. Mean O3 concentrations during the rides measured by these stations varied between 0.033 and 0.103 ppm (mean 0.076 ppm).

Immediately before and after each ride, the subjects provided a blood sample for the assay of serum CC16 and performed respiratory function tests [forced vital capacity (FVC), forced expiratory volume in 1 sec, (FEV1), peak expiratory flow (PEF), and maximum expiratory flows at 25, 50, and 75% of the vital capacity (MEF25/50/75%)]. We assessed subjects’ lung function using a spirometer equipped with a Fleisch pneumotachometer (Fukudasangyo Europe, Bologna, Italy). Subjects were tested within 30 min before and after the ride, while wearing nose clips. Mean FEV1 and FVC values were the means of the two or three best acceptable values tests of lung function, according to the American Thoracic Society (10). The concentration of CC16 in serum was determined by an automated immunnoassay relying on the agglutination of latex particles (11,12). The accuracy of this immunnoassay was recently confirmed by comparison with a monoclonal antibody-based ELISA (13). Cystatin C, a small-size protein like CC16, was determined in serum to detect possible variations in the glomerular filtration rate (14), a potential confounder for CC16 concentrations (7).

Study on mice. Two-month-old female C57Bl/6 mice (Iffa Credo, l’Asbrele, France) were exposed to 0.08 ppm O3 or to filtered air for 4 or 8 hr in inhalation chambers (Sheet Metal Products, Dust Control Systems; Young & Bertke Co., Cincinnati, OH). O3 was produced from dried and filtered air by a high-voltage generator (Anseros Ozomat; Anseros Klaus Nonnenmacher GmbH, Tübingen, Germany) and continually monitored by an ultraviolet photometric analyzer (Signal Instrument Company, Farmington-Oxon, UK). Immediately after exposure, the animals were sacrificed with sodium pentobarbital (100 mg/kg, ip) to collect serum and bronchoalveolar lavage fluids (BALFs). Bronchoalveolar lavage and cell counts were conducted according to the technique described previously (15). Briefly, the lung was washed 3 times with a 2-mL volume of saline, then BALFs were centrifuged for 10 min (1,000 g at 4°C). We used the cell-free supernatant of the first lavage fraction for biochemical measurements, whereas we used the cell pellets of BALFs for total and differential counts. The
BALF pellets were resuspended in 1 ml NaCl containing 0.1% bovine serum albumin, and we determined the total number of live cells by the trypan blue exclusion method. We determined the cell differential counts of macrophages and polymorphonuclear neutrophils (PMNs) by characterizing 200–250 cells/animal on cytocentrifuge preparations fixed in methanol and stained with Diff Quick (Dade Behring AG, Düdingen, Switzerland). We measured the concentrations of CC16 in BALF and serum by an automated latex immunoassay recently developed for rodent CC16 (16). A similar immunoassay was also used for the determination of albumin in BALF (16).

Statistics. The results were expressed as mean ± SE. All statistical tests were applied on log-transformed data. We assessed the differences between pre- and postride values using the paired Student's t-test. Factors significantly influencing serum concentrations of CC16 or changes in lung function tests were identified by stepwise multiple regression analysis. We assessed the differences between values in quartiles of O3 concentrations by one-way analysis of variance followed by the Dunnett's multiple comparison test. In the animal study, we compared mean values of control and O3-exposed groups by the Student's t-test. The level of significance was assigned at p < 0.05.

Results

After the ride, the mean ± SE serum concentration of CC16 was significantly increased in both men (12.3 ± 0.9 vs. 11.2 ± 0.8 µg/L; n = 18, p = 0.011) and women (11.9 ± 1.3 vs. 11.1 ± 0.6 µg/L, n = 30, p = 0.012). In contrast, pre- and postride concentrations of serum cystatin C were similar (1.22 ± 0.22 vs. 1.20 ± 0.21 mg/L, n = 48, p = 0.162).

Stepwise regression analysis of all data shows that the increase in serum CC16 during the ride (i.e., the difference between post- and preride concentrations) was independent of sex and of cystatin C variations in serum (R² = 0.01, p = 0.41), but correlated with the O3 concentrations (R² = 0.18, p = 0.0024). We found an even more significant correlation between O3 levels and the postride concentrations of CC16 in serum (R² = 0.29, p < 0.0001). Interestingly, O3 levels were also correlated with preride serum CC16 concentrations (R² = 0.13, p = 0.011), most probably because the first blood sample was collected when subjects had been exposed to O3 before the exercise. These associations were not confounded by the renal function because serum cystatin C emerged as a significant determinant only for the preride serum CC16 levels (R² = 0.1, p = 0.02).

To examine dose–effect relationships, we divided the subjects into quartiles of increasing O3 levels. Both pre- and postride concentrations showed an exposure-related trend; the rise over the first quartile is significant from the fourth and third quartile onward, for the pre- and postride, respectively (Figure 1). Post-ride CC16 in the fourth quartile was increased by 53% on average as compared to the first quartile. When we compared pre- and postride concentrations of serum CC16 within each quartile, the postride elevation of serum CC16 was statistically significant from the second quartile, corresponding to O3 levels between 0.060 and 0.084 ppm.

The comparison of lung function performances before and after the ride on the whole population did not reveal any significant decrement in the FEV1, FVC, which are the parameters classically impaired by O3. However, the MEF25% (p = 0.021) and PEF (p = 0.006) were slightly decreased. We also found significant correlations between O3 concentrations and decreases in FEV1 (R² = 0.144, p = 0.008), FVC (R² = 0.162, p = 0.005), MEF25% (R² = 0.142, p = 0.032), and MEF75% (R² = 0.09, p = 0.037). When the subjects were classified in quartiles of O3 levels, we found significant decreases in several lung function parameters in quartile 3 (FVC, FEV1, MEF25%, MEF75%, and PEF) and/or quartile 4 (FVC, MEF75%, and PEF) (Table 1). However, changes in lung function parameters were not correlated with those in serum CC16 levels (R² = 0.05, p = 0.15).

We confirmed the ability of O3 to alter the lung epithelial barrier in animals at ambient air levels. Female C57Bl/6 mice were exposed to 0.08 ppm O3 for 4 and 8 hr. We determined CC16 in serum and bronchoalveolar lavage together with classical indicators of lung injury. As shown in Figure 2, O3 produced an increase in serum CC16 that was already statistically significant after 4 hr of exposure. After 8 hr, this increase was more pronounced and was accompanied by an influx of albumin and of PMNs in BALF. At this stage, the inflammatory response was associated with an enhanced bidirectional leakage of proteins across the pulmonary epithelial barrier, which, by light microscopy, appears morphologically intact (results not shown). The level of CC16 in BALF was unchanged.

Discussion

Our study shows that in both humans and mice, short-term exposure to ambient levels of O3 induce an early increase of serum CC16 occurring before most other manifestations of lung toxicity. Because CC16 is synthesized and secreted almost exclusively by the lung Clara cells, its elevation in serum is a non-specific measure of Clara cell injury. By contrast, the increase in CC16 concentration during exercise, already observed in respiratory patients with chronic obstructive pulmonary disease (10), indicates a more specific involvement of these cells during exposure to O3. Additional studies are needed to identify the mechanisms controlling Clara cell secretion of CC16 in response to O3 exposure and to explore further the role of this protein as a biomarker of pulmonary toxicity.
can be explained only by assuming an increased intravascular leakage of the protein across the lung epithelial barrier (5-7, 16).

Another possible explanation is a reduced renal clearance, but such a mechanism can be formally ruled out because in our study the renal function was not impaired by the exercise or by O3. The possibility of an increased synthesis of CC16 causing the elevation of CC16 in serum can also be excluded because the levels of CC16 in BALF in mice were unaffected by O3 exposure.

As reviewed recently by Hermans and Bernard (7), there is now ample evidence that protein transfer across the lung epithelial barrier occurs mainly by passive diffusion through water-filled porous channels in the tight junctions. The increased permeation of proteins across the air-blood barrier observed in lung injury most likely results from a loss of the tight selectivity of the epithelial barrier due to an enlargement of paracellular pores or to the appearance of nonrestrictive transepithelial leaks. Alternate mechanisms such as basolateral secretion or transcellular passage, which have been invoked to account for the increased transepithelial flux of proteins, have not received experimental support. The intravascular leakage mechanism for the elevation of serum CC16 is fully consistent with the current understanding of the epithelial toxicity of O3, considering that the primary effect of O3 on the lung epithelium is an increased permeability due to the enlargement or formation of intercellular channels (9, 17, 18). In mice exposed to 0.08 ppm O3, loss of the size selectivity of the lung epithelial barrier was confirmed by an elevation of albumin in BALF, which is classically interpreted as an evidence of increased permeability to proteins.

In cyclists, both the pre- and postride elevations of serum CC16 showed very significant correlations with the O3 concentrations in air. These correlations were much higher than those emerging between function deficits and O3. The association with O3 was particularly remarkable with the postride values of serum CC16, when the delivered dose of O3 was increased by both the exercise and the higher ambient O3 levels in the afternoon. By contrast, no association was found between the increase of serum CC16 and lung function deficits. This is not surprising because at moderate O3 levels, lung function decrements do not correlate with inflammatory changes in BALF, which usually occur earlier (19-22). This earlier increase of epithelial barrier permeability also clearly emerges from our study because the rise of serum CC16 was statistically significant in quartile 2 (0.0605-0.084 ppm), in contrast to lung function changes, which were significantly altered only from quartile 3 (0.084-0.0925 ppm) or 4 (0.0925-0.103 ppm) (Figure 1). However, the most remarkable finding was that serum CC16 in mice shows a sensitivity to O3 almost identical to that observed in humans. In both species, the protein rose in serum after only a few hours of exposure to average O3 levels around 0.08 ppm (Figure 2). This indicates that the human lung responds to O3 with nearly the same sensitivity as the lung of the C57Bl/6 mouse, a mouse strain among the most sensitive to this air pollutant (23).

Although the exact mechanisms governing the transepithelial passage of CC16 are still poorly understood, we think that the higher sensitivity of serum CC16 to a disruption of the lung epithelial barrier in comparison with BALF albumin mainly stems from the differences in the concentration gradients that drive the diffusion of these proteins across the bronchoalveolar-blood barrier. Figure 3 illustrates schematically the transepithelial leakage of CC16 and albumin across a terminal bronchiule, the principal site of CC16 secretion (7) and also of injury by O3 (9,24). In normal lung (Figure 3A), the epithelium is the main barrier hindering the bidirectional air-blood exchange of proteins; the endothelium offers some resistance only to the passage of proteins the size of albumin or larger (interstitial fluid/plasma albumin ratio estimated at 0.5-0.6) (25,26). Proteins entering the lung interstitium are constantly removed by lymphatic drainage. After acute exposure to O3 (Figure 3B), only the epithelium is damaged; the endothelium remains intact up to levels of 0.7 ppm O3 (27). Under these circumstances, we believe that the leakage of serum CC16 across the epithelial barrier occurs first because it is greatly facilitated by the huge transepithelial concentration gradient of CC16 (around 5,000) as compared to that of albumin (around 2). These gradients are presumably related to the different sizes of the compartments in which leaking proteins are diluted (epithelial lining fluid, 20 mL; CC16 distribution space, 40 L). The small size of CC16 might conceivably make this protein more sensitive than albumin to a slight enlargement of the transepithelial protein pathways induced by O3.

Our study is the first to demonstrate an increased epithelial barrier permeability in humans exposed to ambient O3, by applying a new noninvasive test in a field study. So far, such an effect has been reported only in animals and humans after controlled exposures to O3 in terms of elevation of albumin or total protein concentrations in BALF. In humans, the lowest exposure level of O3 at which this alteration of the epithelial permeability has been observed is 0.1 ppm for 6.6 hr under conditions of moderate exercise (28). In animals, increased permeability occurs from threshold concentrations between 0.1 and 0.2 ppm O3 (24). We found an increased airway permeability in moderately...
exercising subjects exposed on average to 0.07 ppm O₃ during 2 hr. This level, which is below the new 8-hr standard for O₃ in the United States (3), is in the range of the maximum natural levels of ambient O₃ that can be encountered in clean nonurban areas of the United States during the summer season (29). These findings are disturbing because they suggest that natural background concentrations of O₃ have now reached levels above which there is almost no safety margin for the effects on airway permeability. This increase is not so surprising when we consider that tropospheric O₃ has globally increased over the past century by a factor of approximately 3 as compared to preindustrial times (0.01 to 0.015 ppm) (29).

The long-term significance of this altered epithelial permeability caused by O₃ in ambient air is unknown. As shown by animal and human studies using BALF, this phenomenon is a characteristic component of the acute inflammatory response to O₃ that accompanies other inflammatory changes such as leukocyte influx and cytokine release (9,28). Several animal studies suggest that the prolonged maintenance of these effects might be detrimental to the lung tissue. In monkeys, exposure to 0.15 ppm O₃, 8 hr/day, for up to 90 days results in morphologic alterations of the pulmonary epithelium consisting of epithelium thickening and cellular proliferation in the interstitium (30). Similar epithelial lesions have been described in the lungs of rats exposed to an O₃ concentration as low as 0.12 ppm, 12 hr/day, for 6 weeks (31).

In summary, the application of a new noninvasive test to evaluate the permeability of the lung epithelial barrier shows that the pulmonary epithelium is much more sensitive to O₃ oxidative stress than suggested by previous studies. Alterations of the airway epithelium resulting in an increased leakage of proteins into the bloodstream were observed after only 2 hr in moderately exercising subjects exposed to ambient O₃ levels below the new NAAQS standard for O₃ (3). These observations indicate that air pollutants can produce effects on the pulmonary epithelium that are underestimated or undetected using classical tests. Markers of the lung epithelium integrity that are measurable in serum (such as CC16) represent new tools which undoubtably should improve the assessment of health risks from air pollutants and the subsequent derivation of health-based air quality standards.

REFERENCES AND NOTES


Figure 3. Schematic representation of the passage of albumin and CC16 across the different barriers separating the airways from the blood at the level of a terminal bronchiole (A) under normal conditions or (B) after acute exposure to O₃. Abbreviations: En, endothelium; Ep, epithelium; In, interstitium. The thickness of the arrows is proportional to the concentration of the proteins but is used to illustrate the relative permeabilities of the different barriers and the increased fluxes caused by O₃ exposure. The concentrations of albumin in the epithelial lining fluid and blood estimated in normal subjects are approximately 3.5 and 20 g/L, respectively (24). The corresponding values for CC16 are approximately 100 mg/L and 15 pg/L, assuming a free exchange of the protein across the endothelium (7). For CC16, acute exposure to O₃ (B) causes an increased permeability of the epithelial barrier to proteins (9,17), resulting in an increased leakage of plasma albumin in the airways and, in the opposite direction, of CC16 into the interstitium from which it is cleared by lymphatic drainage or directly in the blood across the endothelium.

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