

Inhaled LPS induces blood release of Clara cell specific protein (CC16) in human beings

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Background: Animal models of lung inflammation have validated the plasma 16-kd Clara cell protein (CC16) as a peripheral marker of the permeability of the alveolocapillary barrier.

Objective: We investigated in human beings whether inhaled LPS induced a rise in airways permeability measured by the plasma changes in CC16.

Methods: The CC16 was measured in plasma from 15 subjects exposed to LPS by inhalation, during which the kinetics and the dose-response relationship of LPS-induced CC16 were evaluated. Because LPS-induced response involves macrophages activation, the protective effect of oral methylprednisolone was also evaluated.

Results: An inhalation of 50 µg LPS induced a significant ($P < .001$) rise in CC16 after 6 hours (from $7.24 [\pm 0.68] \mu\text{g/L}$ to $10.69 [\pm 0.99] \mu\text{g/L}$) that normalized at 24 hours ($6.65 [\pm 0.33] \mu\text{g/L}$). The CC16 response was dose-related, with the no-response threshold 0.5 µg LPS. A 6-day treatment with 20 mg/d methylprednisolone inhibited significantly ($P < .001$) the CC16 response to 50 µg LPS.

Conclusion: Exposure to LPS by inhalation in healthy subjects induces an intravascular leakage of CC16 that can be blocked by corticosteroids. These observations further validate plasma CC16 as a noninvasive test of the alveolocapillary barrier permeability. (*J Allergy Clin Immunol* 2005;115:1143-7.)

Key words: LPS, lung permeability, Clara cell protein (CC16)

The Clara cells are localized at the junction between the conducting airways and the gas-exchange area of the pulmonary acinus¹ and are thought to protect the lung against environmental injuries. One of the major proteins secreted by the Clara cells is the 16-kd Clara cell protein (CC16), which plays an important anti-inflammatory role

Abbreviation used

CC16: 16-kd Clara cell protein

in the lung.²⁻⁵ The CC16 protein is also considered a marker to evaluate the integrity of the air-blood barrier.⁶⁻⁸ Various clinical and experimental studies suggest that blood CC16 is a sensitive marker to detect early permeability changes of the lung epithelial barrier and/or to evaluate the integrity of the Clara cells, a privileged target of many pneumotoxicants.⁶⁻⁸

Endotoxin and its purified derivative LPS are gram-negative bacterial potent proinflammatory constituents continuously shed into the environment.⁹ Exposure to endotoxin in house dust has been associated with the risk to develop allergies, asthma, and other pulmonary conditions.¹⁰ In human beings, experimental exposure to LPS induces flulike symptoms and inflammation at the blood and airways levels in which macrophages, neutrophils, and CD8⁺ cells are involved.¹¹⁻¹⁵ In animals, intratracheal LPS is associated with an elevated serum concentration of CC16 in spite of a marked decrease of secretion and synthesis at the lung level, suggesting a passage of CC16 from the airways to the vascular compartment as a consequence of increased airway permeability.¹⁶

The aim of the current study was to investigate whether LPS inhalation in human beings modifies the alveolocapillary permeability by measuring the blood concentration of CC16 as a peripheral marker of the airway permeability. The kinetics of the response, the dose-response relationship, and the protective effect of oral corticosteroids have also been investigated.

METHODS

Populations

In a group of 15 healthy nonsmoking subjects, plasma samples were obtained before, 6 hours after, and 24 hours after exposure to LPS by inhalation. At 1-week intervals, the population was exposed to saline, 0.5, 5, and 50 µg LPS, in random order. The mean age was $31.1 (\pm 3.0)$ years; there were 8 female and 7 male subjects.

Another group of 15 healthy nonsmoking subjects (different from the first group) was exposed to 50 µg LPS after a 6-day pretreatment with placebo and methylprednisolone (20 mg/d) in a double-blind, randomized cross-over design. There was a period of washout of

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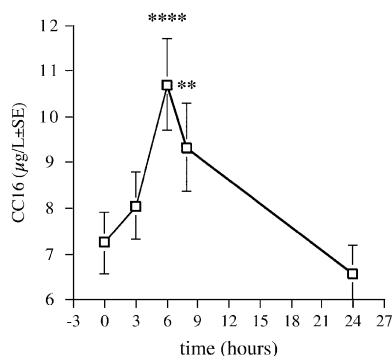


FIG 1. The time-related response of CC16 induced by inhalation of 50 µg LPS. Results are means ($\pm$ SEs). $F_{14,4}$ test = 9.62; $P < .001$. Wilcoxon paired test: ** $P < .02$; **** $P < .001$.

7 days between each treatment period. Plasma samples were obtained before and 3, 6, 8, and 24 hours after exposure to evaluate the CC16 response. This population included 6 male and 9 female subjects (mean age, 36 [$\pm$ 8] years).

The Ethical Committee of the Saint-Pierre University Hospital approved the protocols, and written informed consent was obtained from each subject.

LPS challenge tests

Using a single-blind procedure, all of the subjects were challenged between 8 and 9 PM by inhalation of saline or a dose of LPS (*Escherichia coli* 026:B6, ref L-2654; Sigma Chemical, St Louis, Mo) diluted in saline, after a 3-minute vigorous (vortex) shaking, at a final concentration of 0.1 or 1 mg/mL, immediately before the challenge. The nebulized solutions were administered by a Mefar dosimeter MB3 (Brescia, Italy), delivering a calibrated aerosol of 5 µL per aerosol over a period of 0.6 seconds, at each slow inhalation, starting from the functional residual capacity to the total lung capacity.¹⁷ The jet nebulizer generated heterodispersed droplets of 1 to 4 µm. The 0.5-µg dose corresponded to the no-response threshold, whereas the highest dose of LPS (50 µg) was associated with slight clinical symptoms in some healthy subjects.¹¹

Required blood was collected into EDTA predose and 3, 6, 8, and 24 hours after LPS exposure. Clinical symptoms, temperature, and spirometry (FEV₁) were recorded. A rise of 1.0°C temperature or a decrease in FEV₁ of 10% or more was considered significant.

CC16 measurement

Plasma CC16 was determined by a latex immunoassay using rabbit anti-Clara cell protein antibody (Dakopatts, Glostrup, Denmark) and a standard purified CC16 isolated from human lung lavage.¹⁸ The assay has been validated by comparison with a mAb-based ELISA.¹⁹ All samples were run in duplicate at 2 different dilutions. The between-run and within-run coefficients of variation ranged from 5% to 10%, as reported previously.¹⁸

Statistics

All of the results were expressed as means $\pm$ SEs. The significance of the CC16 response related to the time (factors subject and time) as well as to the dose of LPS or to the inhibiting treatment (factors subject and dose or treatment) was tested by 2-way ANOVA.²⁰ When the F test was significant, unpaired and paired values were tested by the Mann-Whitney and Wilcoxon nonparametric tests, respectively. A P value $< .05$ was considered significant.

RESULTS

The exposure was well tolerated, and no serious adverse reaction was observed. There was no significant change in temperature and/or spirometry. Four subjects developed slight flulike symptoms.

In the basal state, the mean concentration of plasma CC16 for the 30 subjects from the 2 populations was 7.9 (± 0.5) ng/mL, ranging from 2.8 ng/mL to 13.1 ng/mL.

An inhalation of 50 µg LPS induced a significant rise in CC16 plasma concentration. The kinetics of the response, reported in Fig 1, showed no significant change in CC16 concentration 3 hours after LPS exposure, whereas the response was significant after 6 and 8 hours ($P < .001$ and $P < .02$, respectively), returning to the basal value at 24 hours. The peak of the response was observed at 6 hours, with the CC16 rising from 7.24 (± 0.68) to 10.69 (± 0.99) µg/L, ie, an increase of approximately 50%, compared with the basal value. There was a large intersubject variation in the amplitude of the response: among the 15 subjects, the mean increase in CC16 was 3.45 (± 0.64) ng/mL, ranging from -1.25 ng/mL to 8.63 ng/mL.

The peak response of CC16 was related to the level of LPS exposure. Fig 2, A, suggests that the threshold of a significant response in CC16 is 5 µg LPS exposure ($P < .05$). An inhalation of 0.5 µg did not induce a significant change in the CC16 blood concentration. Six hours after a saline inhalation, there was a significant ($P < .01$) decrease in CC16. Compared with the CC16 measured after an inhalation of saline, the response to LPS was related to the inhaled dose (Fig 2, B).

The possible blocking effect of methylprednisolone on the CC16 response to inhaled LPS was then investigated. Compared with placebo, a pretreatment of 6 days with methylprednisolone 20 mg/d significantly abolished the LPS-induced CC16 response (Fig 3). The protective effect of methylprednisolone on the CC16 response measured at 6 hours after LPS inhalation is shown in Fig 4. The basal value of CC16 decreased significantly ($P < .05$) after methylprednisolone compared with the placebo (Fig 3).

DISCUSSION

The current study shows that in healthy subjects, an inhalation of LPS results in a dose-related increase in blood CC16 that peaks at 6 hours and normalizes at 24 hours, and that this response is blocked by a pretreatment with oral methylprednisolone.

Acute lung injury by smoke, ozone, and, recently, nitrogen trichloride (NCl₃) exposure has also been associated with expression of CC16 at the blood level in human beings²¹⁻²³ only a few hours after exposure. Using higher doses of LPS (100 µg/100 g weight) in animal models, the CC16 appears in serum after 3 hours, returning to the basal value after 3 days; at the airways level, there is a decrease in CC16 at 6 hours in the absence of a cytotoxic effect of LPS on Clara cells.¹⁶ This suggests that intravascular leakage of CC16 from the lung to the vascular compartment

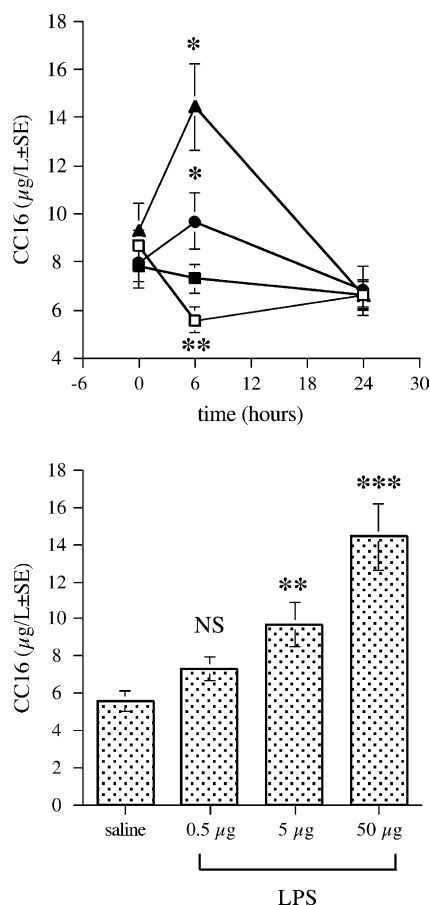


FIG 2. A, The blood CC16 concentrations (means $\pm$ SEs) after an inhalation of saline (□—□, $P < .005$), 0.5 μ g LPS (■—■, NS), 5 μ g LPS (●—●, $P < .002$), and 50 μ g LPS (▲—▲, $P < .0001$). **B,** The dose-response relationship of the peak CC16 values ($P < .0001$). Wilcoxon paired test: * $P < .05$; ** $P < .02$; *** $P < .01$.

is the main mechanism explaining the rise in blood CC16 and the decrease in bronchoalveolar lavage fluid CC16. One could argue that CC16 is produced by other tissues because the response to inhaled LPS involves multiple organs.¹¹ However, in human beings, Northern blot analysis performed on 50 different tissues has shown that CC16 is expressed almost exclusively in the respiratory tract.⁶ In the current study, the LPS-induced rise in blood CC16 in human beings can be considered the consequence of an increase in airways permeability. Of note, Sandstrom et al²⁴ did not observe a significant change in lung permeability by using assessment of albumin in the bronchoalveolar lavage fluid obtained from human subjects exposed to 100 μ g inhaled LPS. However, the lavage fluid was sampled 3 hours after the LPS exposure, which could be too early to observe a change in the lung permeability, as also suggested by the current results showing a significant rise in CC16 at 6 hours after LPS, whereas there was no change after 3 hours. Another possible explanation could be the greater sensitivity of blood CC16 to detect increased permeability

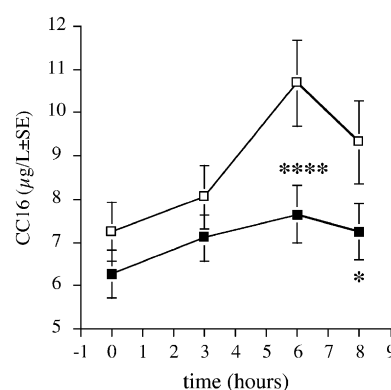


FIG 3. Effects of a 6-day pretreatment with placebo (□—□) and methylprednisolone (■—■) on blood CC16 levels evaluated before and 3, 6, and 8 hours after LPS inhalation. Results are expressed as means ($\pm$ SEs). $F_{4,1} = 36.27$; $P < .0001$. Statistics: Wilcoxon tests. * $P < .05$; *** $P < .001$.

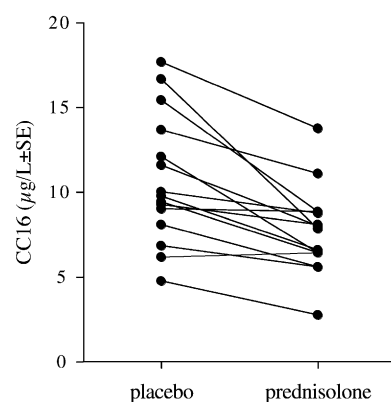


FIG 4. Individual data of the protective effect of prednisolone (20 mg daily for 6 days) on the CC16 measured 6 hours after inhalation of LPS. Statistics: Wilcoxon tests. $P < .001$.

of the epithelial barrier (because of the small size and high transepithelial concentration gradient of CC16 compared with albumin), as suggested in an animal model. Indeed, in mice exposed to ozone, the blood CC16 allows the detection of subtle changes in lung permeability that were not reflected by the levels of albumin in bronchoalveolar lavage fluid.²⁵ Similar observations have been reported after exposure of mice to NCl_3 .⁸

The blood CC16 expression is related to the dose of inhaled LPS. The response is significant after an exposure of 5 μ g. The threshold of the CC16 response was observed for 5 μ g LPS, which is similar to other inflammatory parameters measured in blood or sputum (neutrophilia, acute phase proteins, and TNF).¹¹ This level of exposure is comparable with the estimated lung LPS dose from smoking 2 packs of cigarettes per day (2400 ng LPS/pack), because bacterial endotoxin is an active component of cigarette smoke.²⁶ This level is consistent with the level of LPS exposure of poultry farmers and swine workers at risk of lung diseases.⁹ Given these observations, the monitoring of CC16 levels may give

insight into the disease prevalence or outcome in subjects exposed to smoke/dusts containing LPS in both domestic and occupational environments.

Surprisingly, we have observed a decrease in CC16 six hours after saline inhalation. Because a change in the respiratory permeability is improbable after saline, a possible explanation could be a circadian change in the lung permeability levels, higher at 9 AM than 3 PM. This hypothesis is supported by recent data reporting a spontaneous decrease of the level of blood CC16 in the afternoon compared with the morning in human beings.²⁷ Because of this baseline variation of CC16, Blomberg et al²⁸ concluded that data must be paired, whereas in environmental studies, the responses must be followed longitudinally.

Classically, resident alveolar macrophages were thought to play a critical role in response to inhaled LPS.²⁹ A 6-day pretreatment with methylprednisolone completely inhibited the CC16 response to LPS. The anti-inflammatory properties of corticosteroids are complex because virtually all of the inflammatory and structural cells of the airways are involved. Nevertheless, the monocytes/macrophages are particularly sensitive to corticosteroids, whereas neutrophils are relatively unresponsive.³⁰ Because the CC16 response to LPS is inhibited by a pretreatment with oral corticosteroid, we speculate that the LPS-induced change in the alveolocapillary permeability is, at least in part, mediated by macrophages activation. A transgenic mouse was created expressing a mutant version of the inhibitory protein I- κ B α .³¹ This I- κ B α superrepressor, which acts to repress nuclear factor- κ B action exclusively in the epithelial cells of the airways, is sufficient to blunt the lung responsiveness to intranasal LPS instillation.³¹ Taking into account this last study, we speculate that macrophages probably require airway epithelial cells activation to generate changes in the permeability of the alveolocapillary barrier. There is also a clinical consequence of the inhibiting effect of corticosteroids on the LPS-induced increase in CC16. In acute lung injury, in which there is a change in lung permeability, rapid treatment with corticosteroids could limit the leakage of CC16 from the lung to the vascular compartment. This effect could limit the lung injury, because the CC16 protein appears to protect the respiratory tract against oxidative stress and inflammation.³² This is exemplified by a decrease in blood and airways levels of CC16, which are associated with the risk of bronchiolitis obliterans in lung transplant recipients.³³ The importance of CC16 in the lung as a protective factor is further reinforced by a recent study showing that CC16 gene polymorphism influences the degree of airway responsiveness in children with asthma.³⁴

In animal models, it is well known that intratracheal inhalation of LPS induces an increase in lung epithelial permeability.^{35,36} As a consequence of the change in permeability, plasma extravasation into the airways is a risk factor to develop asthma and hyperresponsiveness to several environmental agents, such as airborne endotoxin and allergens.^{37,38} Boehlecke et al³⁹ observed that a 4-hour exposure to a level of endotoxin that may be

encountered in indoor air enhances the airway response of persons with allergic asthma to inhaled allergen. In children, the response to allergen by nasal challenge is enhanced when allergen extracts are mixed with LPS.⁴⁰ Recently, Tulic et al³⁸ reported that, in ovalbumin-sensitized rats, a pre-exposure to LPS exacerbated the allergic response to ovalbumin, exaggerating the inflammatory cell influx and microvascular leakage into the bronchoalveolar lavage fluid. This effect was significant with the condition that the LPS exposure occurred more than 6 days after the ovalbumin sensitization. Taking these observations into account, we speculate that the LPS-induced rise in lung epithelial permeability (suggested by the results of the current study) could enhance the severity of airways diseases in domestic or occupational settings contaminated by endotoxin.

In human beings, inhaled LPS induces plasma CC16 expression, suggesting an increase in the permeability of the alveolocapillary barrier that, in turn, could sensitize the lung and airways tissues to noxious airborne particles and vapors. This also opens the possibility that blood CC16 monitoring could have prognostic and/or diagnostic potential in evaluating the risk of developing lung injury in subjects exposed to environmental airborne endotoxin.

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