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Exercise alters serum pneumoprotein concentrations

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Abstract

To determine the effect of exercise on serum levels of Clara cell protein (CC16) and surfactant-associated protein A (SP-A), serum was collected from 14 healthy subjects 1 h after maximal and sub-maximal exercise. Healthy volunteers participated on separate occasions in a control (no exercise) session, simulated firefighting tasks for 30 min ($n = 14$), and intermittent treadmill exercise at near maximal heart rates for 60 min ($n = 10$). Serum samples and induced sputum samples were collected 1 h post exercise. Induced sputum fluid was analyzed for tumor necrosis factor alpha (TNF-α), an inflammatory mediator produced by pulmonary macrophages. Serum CC16 levels increased significantly with both firefighting tasks (15 ± 13 μg/L vs. 9 ± 4 μg/L, $P = 0.047$) and treadmill exercise (15 ± 8 μg/L vs. 9 ± 4 μg/L, $P < 0.01$). Serum SP-A concentrations did not change compared to control with either firefighting tasks (247 ± 106 μg/L vs. 247 ± 96 μg/L, $P = 0.84$) or treadmill exercise (251 ± 89 μg/L vs. 285 ± 87 μg/L, $P = 0.44$). TNF-α concentrations in sputum supernatant showed no significant difference from controls. These results show an increase in serum CC16 after exercise. This must be considered when utilizing serum CC16 to determine the presence of lung injury in settings that combine exercise and toxic exposures. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Clara cell protein (CC16) is a 16-kDa protein released from non-ciliated pulmonary epithelial cells (Clara cells) in the lungs. CC16 is also produced in the male urogenital tract, but previous studies indicate that serum levels are due mainly to pulmonary transudates (Bernard et al., 1997). CC16 can also be isolated from pleural fluids, amniotic fluid, ascites fluid, and cerebrospinal fluid (Bernard et al., 1993; De Jongh et al., 1998; Hermans et al., 1998). Because of its low weight and small size, CC16 easily diffuses across the bronchoalveolar/capillary barrier and can be detected in peripheral blood serum (Bernard et al., 1992). The function of CC16 appears to be anti-

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inflammatory. CC16 has been shown to inhibit phospholipase A_2, fibroblast chemotaxis, interferon-γ, and interleukin-2 in vitro (Dierynck et al., 1995; Lesur et al., 1995).

Acute lung injury has been shown to produce a transient increase in serum CC16, most likely due to the disruption of the bronchoalveolar/capillary barrier and the movement of CC16 directly into the blood (Jorens et al., 1995; Hermans et al., 1999). The elevation of serum CC16 has been noted as early as the first hour after an acute insult (Bernard and Van Houte, 1996). Hyperlipidemia and obesity have also shown a positive correlation with serum CC16 levels (Nomori et al., 1996). Chronic conditions, such as smoking, occupational exposure to silica, asbestos, and firefighting have shown decreased serum CC16 levels, well before alterations in pulmonary function tests were observed (Bernard et al., 1994; Nomori et al., 1995). Decrease in serum CC16 appears to be a sensitive marker of chronic pulmonary exposure and disease.

Cystatin C is small protein that is filtered freely at the glomerulus and is not secreted by the renal tubule, making it an effective marker for estimation of glomerular filtration rate (Keevil et al., 1998). Correlating changes in serum CC16 concentration with cystatin C eliminates any bias caused by increases or decreases in GFR, as the kidney is a major route of excretion for CC16.

Surfactant associated protein A (SP-A) is a 36-kDa protein produced by Clara cells and type II alveolar cells, and serves as a stabilizer for pulmonary surfactant (Von Essen et al., 1998). In addition to this support role, SP-A has been shown to exert effects on alveolar macrophages, including upregulation of phagocytosis and nitric oxide production (Blau et al., 1997; Weikert et al., 1997; Wright and Youmans, 1998). Given its increased size, SP-A may be less likely to cross the bronchoalveolar capillary barrier, and serum levels may be less sensitive to inhalation injury.

Tumor necrosis factor alpha (TNF-α) is an inflammatory mediator released by pulmonary macrophages (PM) following toxic exposures. PM release of TNF-α has been associated with increased epithelial permeability and an enhanced inflammatory response (Li et al., 1995). Induced sputum has been used as a non-invasive means of collecting bronchial fluid and cells without the need for bronchoalveolar lavage (Pin et al., 1992). By monitoring TNF-α levels in sputum, any observed changes in lung permeability can be associated with an intrapulmonary inflammatory response as measured by this cytokine.

In order to be effective markers for pulmonary injury, CC16 and SP-A levels must be unaffected by daily activity, or corrected for any fluctuations due to non-toxic events. In many settings, exercise may combine with potentially toxic exposures. Exercise has been shown to induce a systemic leukocytosis, and may also alter cellular production of inflammatory mediators (McCarthy et al., 1991). In addition, previous studies have demonstrated increased production of inflammatory cytokines in the plasma of individuals after exercise of varying intensity (Dufaux and Order, 1989; Echtenacher et al., 1990). The physiologic stress of working at high rates of exertion could alter the production or serum concentrations of CC16, making it difficult to quantify changes due to toxic exposures. This would have significant ramifications for the use of serum CC16 in the study of occupational lung injury. The purpose of this study is to examine the effects of exercise on serum CC16 levels.

2. Methods

This study was approved by the University of Arizona Human Subjects Committee, and all subjects gave informed consent. Non-smoking subjects were recruited from the University of Arizona students and faculty. Subjects were asked to refrain from exercise for at least 2 days prior to testing. In a cross-over design, each subject was asked to report three times, separated by at least 1 week, to perform one of three tasks: control (inactivity), firefighting tasks designed to simulate the physical exertion of firefighting, and treadmill exercise. Each day consisted of the assigned task, a blood draw, and sputum induction. Subjects were asked about recent exercise, respiratory illnesses, and allergy symptoms. Blood pressure, pulse, and tympanic temperature were also recorded.

2.1. Firefighting tasks

Firefighting tasks were performed at the Tucson Fire Department training facility. These tasks were designed by the Tucson Fire Department as a pre-employment test of physical capability. Tasks were designed to provide a realistic simulation of the effort involved in actual firefighting work. Each subject wore full turnout gear and airpack, weighing a total of 15 kg, leather gloves, and a bicycle helmet. The subject then completed the following tasks: (1) carrying a 10-kg rolled fire hose and a 5-kg shoulder pack up a fire tower to the fourth floor landing and descending; (2) walking to a fire hydrant with a permanently attached wrench and rotating the wrench five full revolutions against 135 N m of torque; (3) pulling 60 m of fire hose 30 m; (4) removing a ladder from a truck, carrying it to a wall 6 m away, placing the ladder on ladder hooks, extending a nearby ladder 4 m and lowering it again, removing the ladder from the wall hooks and placing it back on the truck; (5) pulling a 60-kg sled by attached drag handles for 50 m. Subjects were instructed to complete these tasks at a comfortable pace, and to continue rotating through the tasks for 30 min. Heart rate was obtained at the completion of the first three cycles and at 30 min.

2.2. Treadmill exercise

Each subject was asked to complete 1 h of treadmill exercise designed to achieve maximal heart rate. Subjects' pulse, blood pressure, and baseline temperatures were recorded prior to exercise. The exercise consisted of 4 min walking at a 7% grade, 2 min of running, and 4 min of seated rest. Running speed was adjusted to obtain predicted maximal heart rates (220 − age). Pulse was recorded after each running segment and at the completion of the rest period, but was monitored continuously during exercise. Each subject completed six 10-min cycles for a total of 60 min. Tympanic temperature was obtained immediately at the end of exercise testing using an Ototemp™ 3000 Infrared Tympanic Temperature Scanner (Exergen Corporation, Watertown, MA)

2.3. Serum pneumoprotein and cystatin analysis

Thirty milliliters of blood was obtained 1 h post-exercise from each subject by venipuncture and collected in uncoated tubes. Each sample was allowed to clot for a minimum of 8 h at 4 °C. Samples were then centrifuged at 3000 rpm for 10 min. Serum was decanted and stored at −20 °C until CC16, SP-A, and cystatin C analysis was completed. CC16 and cystatin C were measured by Latex immunoassay-LIA, while SP-A was measured by competitive ELISA.

2.4. Sputum induction

Sputum was induced by inhalation of 3% hypertonic saline 1 h after completion of fire tasks or exercise. Saline was administered with an ultrasonic nebulizer set at maximal output (DeVilbiss Ultra-Neb 99HD, Somerset, PA) through an adult facemask. Sputum was expectorated into sterile conical vials. Subjects inhaled the saline for 30 min, or until 5 ml of sputum was obtained. Each subject was instructed to cough deeply every 3–5 min. Sputum samples were stored at 4 °C and transported to the lab for immediate analysis.

2.5. Sputum analysis

Cells were isolated according to the method of Von Essen et al. (1998). Each sample was combined with 10 ml of 10% dithiothreitol solution, prepared by mixing 10 ml dithiothreitol (sputolysin; Calbiochem, San Diego, CA), 90 ml HEPES buffered solution (140 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 5 mM glucose, and 10 mM HEPES) and 0.5 ml penicillin/streptomycin (Sigma). Samples were placed in a shaking water bath for 15 min, then vortex mixed. Each sample was filtered through sterile nylon mesh to remove any remaining mucous or particulate matter. Samples were centrifuged at 1500 rpm for 15 min. Two milliliters of supernatant were removed and frozen at −80 °C until time of TNF-α analysis. Supernatant was removed from the cell plug and discarded. Cell plugs were diluted with 1 ml HEPES buffer, and cell counts were obtained with a Neubauer Hemacytometer. Viability was ver-

ified by trypan blue exclusion. TNF-α samples were analyzed by a colorimetric ELISA method (Quantikine HS, R&D Systems, Minneapolis, MN). Plates were read at 490 nm with dual wavelength correction at 690 nm on a spectrophotometric plate reader.

2.6. Statistical analysis

CC16 levels were compared with a two-tailed paired *t*-test for each task. Pearson's *r* was used to correlate changes in serum CC16 with cystatin C to determine the need for glomerular filtration rate (GFR) correction. Two-tailed paired *t*-tests were also used to compare SP-A values for each task, as well as TNF-α concentrations.

3. Results

Twelve male and two female non-smoking volunteers were recruited for the study. All subjects participated as controls and in the firefighting exercises. Ten of the 14 subjects also participated in the treadmill testing. Of all subjects, only four exercised regularly, and three were suffering from seasonal allergies on one or more days of the study. No subjects were experiencing symptoms of respiratory illness during the study. The characteristics of the subjects and the study design are shown in Table 1. Percentage of maximum heart rate achieved during treadmill exercise was near maximal (98% ± 5%), and those achieved during fire tasks were submaximal (83% ± 7%). Mean temperature elevation with treadmill exercise was 0.68 ± 0.35 °C.

Table 1
Study design and subject characteristics

	Day 1	Day 8	Day 15	Day 22
Control	3M[a]	2M	5M/1F	2M/1F
Fire tasks	7M/1F[a]	5M/1F		
Treadmill		4M	5M/1F[a]	

[a] One subject was suffering from seasonal allergies. F, female; M, male.

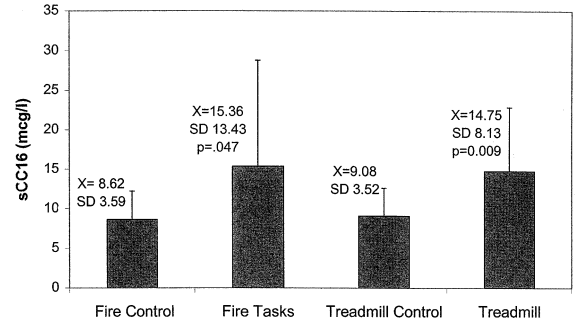

Fig. 1. Serum CC16 concentrations for fire tasks and treadmill exercise compared with controls.

Serum CC16 levels increased significantly after fire tasks (15 ± 13 μg/L) as compared with controls (9 ± 4 μg/L, $P = 0.047$), and were significantly increased after treadmill exercise (15 ± 8 μg/L vs. 9 ± 4 μg/L, $P < 0.01$) (Fig. 1). Pearson's *r* for changes in serum CC16 and serum cystatin C with treadmill exercise and fire tasks indicated no correlation between variables (−0.27 and 0.08, respectively). In addition, there was no statistically significant correlation between serum CC16 and serum cystatin C concentrations following fire tasks or exercise, or in the control groups. Serum cystatin C for fire tasks (901 ± 108 μg/L) was moderately increased over control values (817 ± 108 μg/L), but did not reach statistical significance ($P = 0.07$). Treadmill cystatin C (908 ± 94 μg/L) was significantly increased over control values (787 ± 111 μg/L, $P < 0.01$). Serum CC16/cystatin C increased only with treadmill exercise (0.016 ± 0.008 vs. 0.011 ± 0.004, $P = 0.03$). SP-A following the fire tasks (247 ± 106 μg/L) showed no significant change as compared with controls (247 ± 96 μg/L, $P = 0.84$). Post-treadmill SP-A concentrations (251 ± 89 μg/L) similarly showed no significant difference from controls (285 ± 87 μg/L, $P = 0.44$) (Fig. 2).

TNF-α concentrations in sputum supernatant are shown in Fig. 3. TNF-α during fire tasks (0.078 ± 0.022 pg/ml) showed no significant difference from controls (0.083 ± 0.035 pg/ml, $P = 0.69$). Treadmill exercise TNF-α concentrations (0.079 ± 0.028 pg/ml) were not statistically elevated as compared with controls (0.086 ± 0.040 pg/ml, $P = 0.42$). Mean sputum cell count for

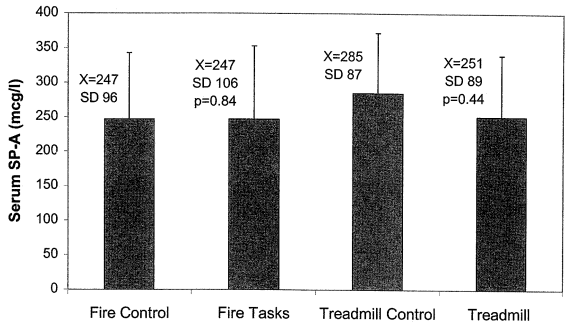

Fig. 2. Surfactant associated protein A concentrations for fire tasks and treadmill exercise.

controls was $3.01 \times 10^6 \pm 2.93 \times 10^6$ cells/sample with no significant difference between groups. Differential counts averaged 90% macrophages, 6% lymphocytes, and 4% neutrophils. Epithelial cells were disregarded. Cell viability was greater than 50% in all samples.

4. Discussion

This study found a transient increase in serum CC16 1 h following both firefighter tasks and near-maximal intermittent treadmill exercise. Increases in serum CC16 concentrations with exercise were not correlated with changes in serum cystatin C concentration, suggesting that mechanisms other than reduced GFR were responsible. SP-A levels showed no significant change with either form of exercise. In addition, sputum supernatant TNF-α concentrations were not affected by exercise.

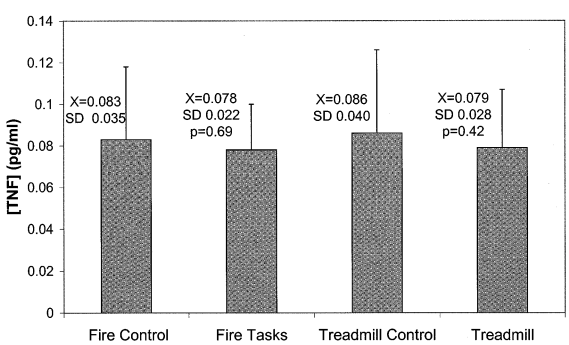

Fig. 3. Tumor necrosis factor alpha concentrations in sputum supernatant after fire tasks and treadmill exercise.

Serum CC16 increased with both modes of exercise, despite differences in intensity and duration. While the estimation of exercise intensity by heart rate is inaccurate for $V\dot{O}_2$ estimation, it can provide an accurate gauge of intensity in the same subject (Sothmann et al., 1991, 1992; Veldhuizen et al., 1996). Using this general criteria, the treadmill exercise required greater effort, while the fire tasks were longer duration at submaximal energy expenditures. Exercise and mild heat stress resulting from exercise have been shown to induce a hormone-mediated systemic leukocytosis and production of heat shock proteins, which may affect cytokine regulation in vivo (McCarthy et al., 1991; Ryan et al., 1991).

There are several potential explanations for the increase in serum CC16 after treadmill exercise and fire tasks as demonstrated in our study. The results may be due to increased alveolar/capillary permeability with effects of exercise limited to small molecules only, since SP-A (36 kDa) serum concentrations did not increase. Alternatively, pulmonary concentrations of CC16 may increase with exercise, with the increased serum CC16 levels explained by the larger diffusion gradient. Changes in serum CC16 were not apparently affected by altered GFR, as there was no correlation demonstrated between serum CC16 and serum cystatin C changes (Doyle et al., 1998). Cystatin C levels in all subjects' control serum were comparable to published population averages, indicating adequate kidney function in all subjects (Erlandsen et al., 1998).

TNF-α concentrations in induced sputum supernatant did not increase with exercise. However, macrophages retrieved from induced sputum may be more mature and less responsive than alveolar macrophages. Therefore TNF-α may have increased at the alveolar level, but not in the airways. One study suggests that these cells from the upper airway are functionally different, and less responsive to external stimuli, such as toxic exposures and stress to the system, although phagocytosis activity was nearly identical to alveolar macrophages (Lehnert et al., 1990). However, production of Interleukins 4 and 5 was found to be nearly identical in cells from induced sputum and cells from bronchoalveolar lavage, suggesting

that some functions of induced sputum cells are not impaired (Olivenstein et al., 1999). In addition, TNF-α is a single inflammatory cytokine, and the lack of change with exercise does not rule out other inflammatory changes.

Serum CC16 has not previously been found to change with exercise. In a study of cyclists exposed to ozone, no increase in serum CC16 was found with exercise alone (Broeckaert et al., 2000). However, the cyclists were well-trained individuals exposed to sub-maximal exercise and varying levels of atmospheric ozone. In a current unpublished study of firefighters and police in Phoenix and Tucson, Arizona, exercise resulted in a significant increase in serum CC16 in police but not firefighters. The exercise by the police appeared to have been more strenuous, and there may have been a differential in level of fitness in firefighters and police. It is possible that serum CC16 may only increase in subjects that do not regularly exercise, as was true with the majority of subjects in our study, or only with high exertion levels.

Further limitations to this study include the lack of data on the kinetics of elimination of CC16, and the lack of data regarding sputum CC16 levels. Without a knowledge of the biologic half-life of CC16, we cannot be sure that the levels obtained are the true peak values, or that the 7-day rest period was sufficient to return the subjects' CC16 concentration to baseline values. However, previous studies have indicated that serum CC16 levels do return to baseline within 10 days of an acute lung injury that raises serum CC16 to 300% of baseline. In addition, based on the size of CC16, its half-life can be estimated to be no more than a few hours in the serum (Bernard et al., 1997). Based on this data, it seems reasonable to expect that CC16 levels will return to normal within a 7-day period with only the relatively mild elevations seen in our study. The source of the increased serum CC16 is assumed to be from the alveolar space, as Clara cells likely secrete the protein into the bronchiolar lumen. However, without sputum CC16 levels, we cannot state this with certainty. Previous studies have indicated that serum CC16 levels are directly correlated with alveolar levels, as measured by bronchoalveolar lavage (Bernard, et al., 1992). This would support our hypothesis that the increases in serum CC16 seen with our study are reflections of increased alveolar/capillary permeability or an increased concentration gradient, and not from direct secretion into the interstitial space. Further studies are needed to elucidate the true nature of the increase in serum CC16.

In summary, the increased serum CC16 seen in near-maximal intermittent treadmill exercise may be due to increases in alveolar–capillary permeability limited to small proteins, increased pulmonary concentration of serum CC16 during exercise, or other unexplained causes. Submaximal, sustained exercise also significantly increased serum CC16. TNF-α concentrations in sputum supernatant indicated no increase in inflammatory activity that could lead to increased permeability, although other inflammatory changes could not be ruled out. Further study is needed to verify and quantify potential changes in serum pneumoproteins with exercise and the mechanisms behind them. Regardless, these increases must be considered when utilizing serum CC16 levels to quantify acute subclinical lung injury in occupations that combine intermittent exercise and toxic exposures. To avoid the confounding effects of exercise, we suggest that serum CC16 samples be collected after refraining from exercise for at least 24 h whenever possible.

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