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Reactive oxygen species in sustained airway constriction induced by citric acid aerosol inhalation

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Abstract

We tested if there is a direct relationship between reactive oxygen species and citric acid-induced airway constriction. Guinea pigs were divided into two groups: control and dimethylthiourea (a hydroxyl radical scavenger). The animals in each group were further separated into four subgroups: baseline, recovery 2–3 min, recovery 10 min, and recovery 20 min. Each animal was anesthetized, cannulated, paralyzed, and artificially ventilated. Citric acid aerosol inhalation caused the following significant changes in the control group during the recovery period: airway constriction for at least 20 min, increases in luminol-amplified *t*-butyl hydroperoxide-initiated chemiluminescence counts in the bronchoalveolar lavage samples at 2–3 and 20 min, an increase in bronchoalveolar lavage fluid substance P level at 2–3 min, and elevations in the bronchoalveolar lavage fluid total cell and neutrophil numbers at 20 min. All citric acid-induced alterations were prevented by dimethylthiourea pretreatment. These results suggest that citric acid inhalation induces the initial release of reactive oxygen species and tachykinins, which causes further cellular infiltration and sustained airway constriction.

Keywords: Airway reactivity; Chemiluminescence count; Inflammatory cell; Tachykinin

1. Introduction

Citric acid aerosol inhalation induces airway constriction, which is mediated mainly via the tachykinin neurokinin$_2$ (NK_2) receptor (Satoh et al., 1993). In addition, we found that reactive oxygen species play an important role in this type of non-cholinergic airway constriction (Lai et al., 1999). However, it is not clear what the actual relationship is between tachykinins and reactive oxygen species.

Furthermore, citric acid aerosol inhalation induces a sustained airway constriction, which is maintained for over 20 min (Lai et al., 1999). However, the airway constriction induced by neurokinin A (the agonist for the tachykinin NK_2 receptor) or by capsaicin (the agent to release tachykinins) in vivo usually lasts less than 10 min (Fujii et al., 1991). Consequently, it is not clear what the mechanism(s) is (are) for the sustained airway constriction following citric acid aerosol inhalation. In this study, we explored the temporal changes in citric acid-induced airway constriction, tachykinin (substance P) level, oxidative stress index (chemiluminescence counts), and alveolar cellular infiltration. We hope to elucidate the underlying mechanism(s) for the citric acid-induced sustained airway constriction.

2. Materials and methods

The study was conducted according to the Guidelines of the American Physiological Society and was approved by the Animal Care and Use Committee of the National Taiwan University.

2.1. Animal preparations

Seventy-three young Hartley strain guinea pigs weighing 213 ± 2 g were divided into two groups: control ($n = 41$) and dimethylthiourea ($n = 32$). No animal in the control group was treated. We demonstrated previously that intraperitoneal injection of saline did not affect airway function or reactivity (Lai and Huang, 2002). Therefore, we did not treat the

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control group with saline. Dimethylthiourea, a scavenger for hydroxyl radicals (Fox, 1984), was dissolved in saline and given to the animals in the dimethylthiourea group for 3 days prior to the study. The three consecutive daily intraperitoneal doses of dimethylthiourea were 250, 250, and 250 mg kg^{-1} (Lai, 1990).

On the day of the study, each animal was anesthetized with sodium pentobarbital (30–40 mg kg^{-1}). Subsequently, each animal's trachea, carotid artery, and jugular vein were cannulated. After being paralyzed with gallamine triethiodide (4 mg kg^{-1}), the animal was artificially ventilated. To ensure that the animal was anesthetized during paralysis, gallamine was administered according to the following plan. Gallamine was only given when its active period (40 min) was within the effective duration of pentobarbital (1–2 h). If it was necessary to administer gallamine beyond this effective period of the anesthetic, supplemental doses of pentobarbital were given before any further gallamine treatment.

Furthermore, the animals in each group were separated into the following four subgroups: baseline, 2–3 min of recovery, 10 min of recovery, and 20 min of recovery. In the first subgroup, all animals were studied after the saline aerosol inhalation, but with no citric acid aerosol inhalation. For the second, third, and fourth subgroups, the animals were first exposed to saline aerosol. Right after the saline aerosol inhalation, the baseline airway function was determined. Then, the animals were exposed to citric acid aerosol, and airway function was examined 2–3, 10, and 20 min following the citric acid aerosol inhalation. For saline or citric acid aerosol inhalation, each animal received 50 breaths of 4 ml saline aerosol or citric acid aerosol generated from a 0.6 M citric acid solution, respectively. Both aerosols were generated from a nebulizer (Ultra-Neb99, DeVilbiss, Somerset, PA, USA).

2.2. Evaluation of bronchial function

Each anesthetized-paralyzed and ventilated animal was placed supine inside a whole-body plethysmograph. The flow rate was monitored with a Validyne DP45 differential pressure transducer as the pressure dropped across three layers of 325-mesh wire screen in the wall of the plethysmograph. Lung volume change was obtained via integration of flow. The airway opening pressure or the arterial blood pressure was measured with a pressure transducer (DTX/Plus, Viggo-Spectramed, Oxnark, CA, USA) connected to a side arm of the tracheal tube or to the arterial catheter, respectively. All of the above signals were recorded (recorder, TA11, Gould, Valley View, OH, USA). During artificial ventilation, tidal volume was divided by its accompanying airway opening pressure difference to obtain dynamic respiratory compliance. The airway opening pressure difference was measured between end-inspiration and end-expiration (i.e., instance of no flow). The full maximal expiratory flow-volume maneuver was performed with inflation of the lung to total lung capacity (the lung volume at Pao = 30 cm H_2O) and subsequent deflation to residual volume with a negative pressure of 40 cm H_2O, which produces the maximal expiratory flow. Maximal expiratory flow at 30% baseline total lung capacity ($\dot{V}max_{30}$) was measured according to our previous method (Lai, 1988). Dynamic respiratory compliance and $\dot{V}max_{30}$ were used as indicators of airway constriction. The general experimental protocol was that the values of dynamic respiratory compliance and $\dot{V}max_{30}$ were obtained after saline, or 2–3, 10, or 20 min after citric acid aerosol inhalation.

2.3. Collection of bronchoalveolar lavage fluid for determinations of chemiluminescence and substance P

Right after the bronchial functional evaluation, 4 ml warm (37 °C) saline containing thiorphan (10^{-4} M) was instilled into the lung of the anesthetized animal via the trachea. The fluid in the bronchoalveolar space was withdrawn 30–40 s after the instillation. Then, 0.5 ml of the collected bronchoalveolar lavage was immediately wrapped with aluminum foil and kept in the ice box until testing for chemiluminescence, which was usually done within 2 h. The rest of bronchoalveolar lavage was centrifuged (3000 rpm) for 10 min. Then, the supernatant was collected and stored at −70 °C for later analysis of substance P.

2.3.1. Measurement of luminol-amplified, t-butyl hydroperoxide-initiated chemiluminescence

Detection of luminol-amplified, *t*-butyl hydroperoxide-initiated chemiluminescence in bronchoalveolar lavage fluid was performed with the method described by Sun et al. (1998) with some modifications. In 0.2 ml bronchoalveolar lavage fluid, 0.4 ml luminol was added to produce a final concentration of 1.8×10^{-4} M to enhance the reactive oxygen species production (Li et al., 1999). The mixture was incubated at 37 °C for 10 min. Then, the changes in chemiluminescence potency (mV) were continuously monitored in the bronchoalveolar lavage sample for a 60-s time period in a sealed dark chamber of the Chemiluminescence Analyzing System (Bio Orbit 1251), which was connected to a computer using a specialized software program (Bio Orbit, Turku, Finland). Subsequently, 0.1 ml of *t*-butyl hydroperoxide (Sigma, St. Louis, MO, USA) in physiological buffer solution (pH = 7.4) was injected into the cell. The chemiluminescence in the sample was measured continuously for a total of 300 s. Integrating the area under the curve and subtracting it from the luminol-initiated chemiluminescence resulted in the calculated total amount of chemiluminescence.

2.3.2. Determination of substance P

Thawed bronchoalveolar lavage samples were purified by filtration through a C-18 column. Then, we measured the substance P levels by solid-phase enzyme immunoassay

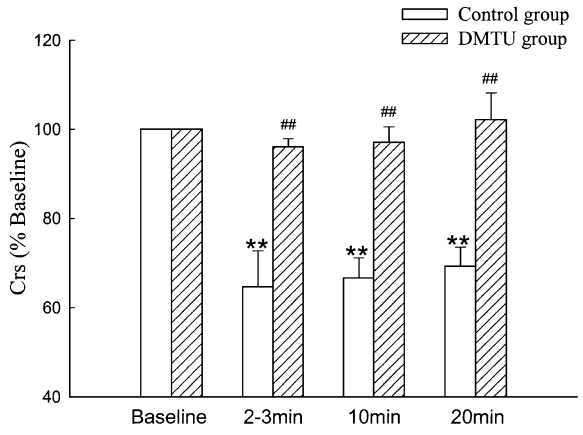

Fig. 1. Values for dynamic respiratory compliance (Crs), expressed as percent of baseline values, at baseline (saline) and at 2–20 min after citric acid aerosol inhalation in two groups of guinea pigs. DMTU, dimethylthiourea. **Significant difference ($P<0.01$) compared to the baseline subgroup. ##Significant difference ($P<0.01$) compared to the control group.

(Lai et al., 1998) with a commercial kit (Cayman Chemical, Ann Arbor, MI). This method has a sensitivity of 12.8 fmol ml^{-1} with both intra- and inter-assay coefficients of variance less than 10%. Dimethylthiourea did not interfere with the substance P assays.

2.4. Bronchoalveolar lavage cell counts

An additional 30 animals were used for bronchoalveolar lavage cell counts. The animals were evenly divided into two groups: control and dimethylthiourea. The animals in each group were further evenly divided into three subgroups: baseline (saline), 2–3 min recovery, and 20 min recovery. Procedures for animal treatments were the same as those described above: 2 ml warm (37 °C) saline was instilled into the lung of the anesthetized animal via trachea at an appropriate time after saline or citric acid aerosol inhalation. The fluid in the bronchoalveolar space was withdrawn 30 s after the instillation. This was repeated 10 times. Pooled bronchoalveolar lavage fluid was centrifuged at 4 °C for 10 min, and the cell pellet was resuspended in Hank's balanced salt solution; 100 μl of Turk's solution was added to the same volume of cell suspension. After mixing, 10 μl of the mixture was placed in a hemacytometer for total cell counts. Differential cell counts were determined from cytospin preparations stained with Liu stain (Handsel Technologies).

2.5. Statistical analysis

All data are reported as means ± S.E.M. One-way analysis of variance was used to evaluate differences among groups or subgroups. If significant difference existed among groups or subgroups, the Newman–Keuls test was used to differentiate differences between any two groups or subgroups. To analyze bronchoalveolar lavage fluid cell counts, the Duncan multiple range test was used instead of the Newman–Keuls test to evaluate differences between any two groups or subgroups. A difference was considered significant when $P<0.05$.

3. Results

3.1. Citric acid-induced airway constriction

For the control and the dimethylthiourea groups at the baseline period, the values for dynamic respiratory compliance were 0.23 ± 0.01 and 0.24 ± 0.01 ml cm H_2O^{-1}, respectively, whereas the values for $\dot{V}max_{30}$ were 41.4 ± 2.0 and 46.4 ± 1.2 ml s^{-1}, respectively. No significant difference in either dynamic respiratory compliance or $\dot{V}max_{30}$ between the two groups at baseline was found. At 2–3 min following citric acid aerosol inhalation, marked decreases in dynamic respiratory compliance (Fig. 1) and $\dot{V}max_{30}$ (Fig. 2) were found, indicating citric acid-induced airway constriction. However, these changes in functional parameters decreased gradually with time. At 20 min after citric acid, dynamic respiratory compliance and $\dot{V}max_{30}$ recovered to about 68% and 75% of the baseline value, respectively. In addition, citric acid-induced airway constriction was significantly attenuated by dimethylthiourea for the whole experimental period.

3.2. Citric acid-induced changes in chemiluminescence counts

Chemiluminescence data are shown in Fig. 3. At 2–3 and 20 min following citric acid, bronchoalveolar lavage chemiluminescence counts increased significantly in the control group. The citric acid-induced increases in chem-

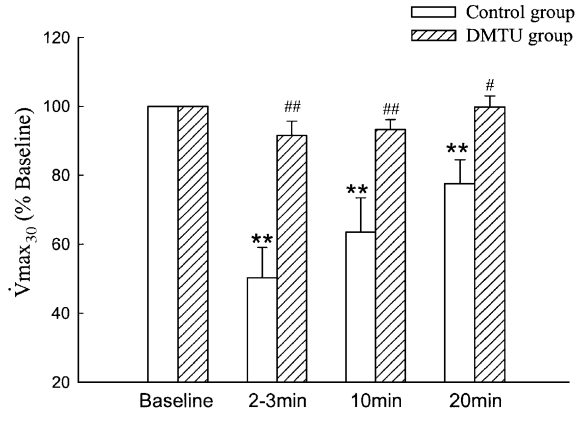

Fig. 2. Values for maximal expiratory flow at 30% baseline total lung capacity ($\dot{V}max_{30}$), expressed as percent of baseline values, at baseline (saline) and at 2–20 min after citric acid aerosol inhalation in two groups of guinea pigs. DMTU, dimethylthiourea. **Significant difference ($P<0.01$) compared to the baseline subgroup. Significant difference compared to the control group: #$P<0.05$; ##$P<0.01$.

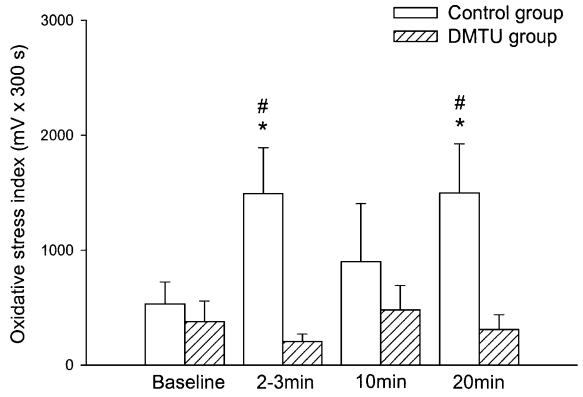

Fig. 3. Values for oxidative stress index (integration of potency over 300 s) at baseline (saline) and at 2–20 min after citric acid aerosol inhalation in two groups of guinea pigs. DMTU, dimethylthiourea. * Significant difference ($P<0.05$) compared to the baseline subgroup. #Significant difference ($P<0.05$) compared to the dimethylthiourea group.

iluminescence counts were significantly attenuated by dimethylthiourea pretreatment.

3.3. Citric acid-induced alterations in bronchoalveolar lavage fluid substance P levels

At 2–3 min after citric acid inhalation, there was a significant increase in bronchoalveolar lavage fluid substance P level (Fig. 4). However, this increase was attenuated in the dimethylthiourea + citric acid group.

3.4. Citric acid-induced alterations in bronchoalveolar lavage cell numbers

None of the cell numbers in the bronchoalveolar lavage samples changed significantly until 20 min following citric acid aerosol inhalation (Fig. 5). At 20 min after citric acid, numbers of total cells, neutrophils, and lymphocytes increased significantly in the control group. These citric

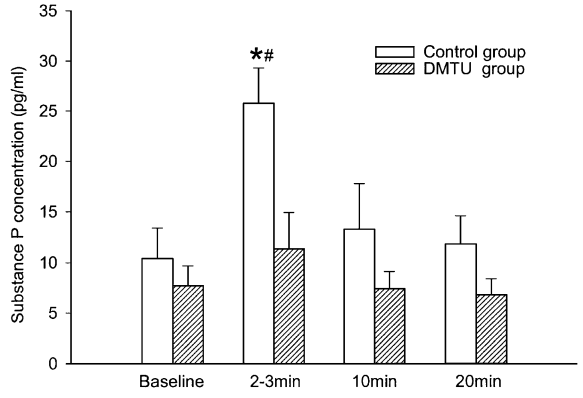

Fig. 4. Concentrations of bronchoalveolar lavage substance P at baseline (saline) and at 2–20 min after citric acid aerosol inhalation in two groups of guinea pigs. DMTU, dimethylthiourea. * Significant difference ($P<0.05$) compared to the baseline subgroup. #Significant difference ($P<0.05$) compared to the dimethylthiourea group.

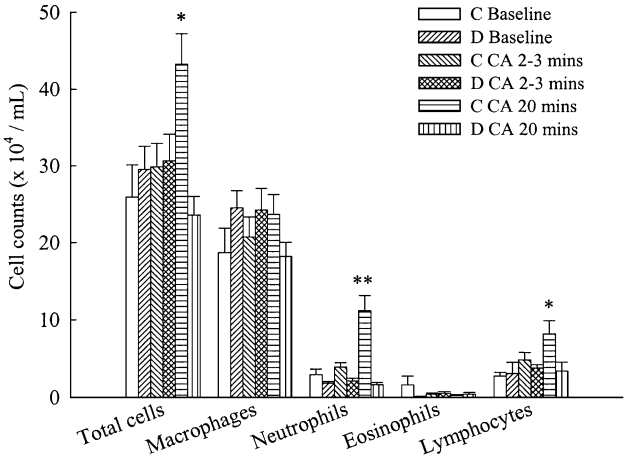

Fig. 5. Bronchoalveolar lavage cell counts at baseline (saline) and at 2–20 min after citric acid aerosol inhalation in the control (C) and dimethylthiourea (D) groups of guinea pigs. CA, exposure to citric acid aerosol. Significant difference compared to all five other subgroups: * $P<0.05$; ** $P<0.01$.

acid-induced increases in cell counts were significantly attenuated by dimethylthiourea.

4. Discussion

At 2–3 min following the challenge, citric acid aerosol induced significant airway constriction and increases in chemiluminescence counts and substance P level in the bronchoalveolar lavage fluid. The airway constriction and increased chemiluminescence counts, but not the substance P level, persisted until the end of the study. However, increases in inflammatory cells were found only at 20 min following the citric acid treatment. Several features of these results will be discussed below.

Satoh et al. (1993) demonstrated that citric acid-induced airway constriction was mediated via tachykinins in guinea pigs. Based on our experience with the relationship between reactive oxygen species and tachykinin-induced non-cholinergic airway constriction, we reasoned that reactive oxygen species might mediate tachykinin release. To explore whether reactive oxygen species mediate tachykinin release, we thus carried out this time study. We found that citric acid caused marked increases in substance P and chemiluminescence counts 2–3 min into the recovery period. These increases occurred simultaneously with a marked airway constriction. Thus, during this initial period, it is possible that citric acid releases reactive oxygen species which, in turn, induce the release of tachykinins. Subsequently, there was a sustained increase in reactive oxygen species levels until 20 min after the citric acid challenge. In this subsequent period, however, the substance P level decreased to near the baseline level. Accordingly, there seems no longer to be a linear relationship between substance P and reactive oxygen species following the initial period.

The increased substance P level in the initial period might induce the sustained elevation in reactive oxygen species during the subsequent period. Brooks and Whelan (1999) and Brooks et al. (1999) demonstrated that substance P, via the tachykinin NK_1 receptor, induced the dose-related production of reactive oxygen species in peritoneal mast cells. Murris-Espin et al. (1995) showed that substance P or its C-terminal amino acids stimulated alveolar macrophages to produce reactive oxygen species. In addition, Sterner-Kock et al. (1999) proved that substance P stimulated human neutrophils, via calmodulin-dependent NADPH oxidase, to produce hydrogen peroxide and nitric oxide.

During the subsequent period, there was still a sustained airway constriction, although there was no elevated substance P level. Therefore, mediators other than tachykinins might cause this subsequent airway constriction. It is tempting to speculate that, due to the sustained elevation in reactive oxygen species, reactive oxygen species might induce the release of constrictors other than tachykinins. It is possible that increased reactive oxygen species might activate phospholipase A_2 (Ryfeldt et al., 1993; Southorn and Powis, 1988) which, in turn, leads to the production of thromboxane, leukotrienes, and/or epoxyeicosatrienoic acids (Reilly et al., 1998; Seeds and Bass, 1999). Then, these increased eicosanoids can induce the observed airway constriction (Holtzman, 1991).

Prior to 20 min following citric acid challenge, no apparent increase in cell counts was found in bronchoalveolar lavage samples. Accordingly, most of the observed increases in oxidative stress have originated from cells other than inflammatory cells. At 20 min after citric acid challenge, significant increases in bronchoalveolar lavage neutrophils and lymphocytes were noted. Therefore, these latter increases in chemiluminescence counts (Fig. 3) could simply be explained by the fact that there are more alveolar cells. This is based on the report of Lu et al. (1996) that chemiluminescence is detectable only in heparinized blood but not in serum. Therefore, chemiluminescence in the present study should also have originated from the cells contained in the bronchoalveolar lavage fluid. Our data are compatible with the fact that neutrophils are an important source of reactive oxygen species (Ryfeldt et al., 1993). No matter where the reactive oxygen species originated, dimethylthiourea pretreatment suppressed the citric acid-induced increases in reactive oxygen species, tachykinins, and airway constriction. This may imply that scavenging hydroxyl radicals might be effective to prevent the citric acid-induced serious chain reactions in vivo.

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