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Silica Complexed with Fe^{3+} Does not Influence Pulmonary Inflammation and Injury*

Jihee Lee · Woul Suh · Eunleung Han
Department of Physiology, College of Medicine, Ewha Womans University

=국문초록=

폐염증 및 손상에 미치는 표면철이 결합된 유리규산의 영향

이화여자대학교 의과대학 생리학교실
이지희 · 서 울 · 한은령

배 경 : 유리규산(silica)에 결합되어있는 표면 철이 Fenton reaction에 의하여 유리규산으로 인한 폐 반응을 증폭시킬 수 있다는 가설을 입증하고자 연구하였다.

방 법 : 생리식염수, silica-H^+, 또는 silica-Fe^{3+}를 기관내 투여한 4시간 후에 폐손상 정도와 호중구의 폐포내 유입을 기관지폐포세척액에서 단백질양과 호중구수를 측정하여 평가하였고, 폐포대식세포 배양액에서 nitric oxide 생성을 nitrate assay로 측정하였다. 또한 silica-H^+, 또는 silica-Fe^{3+}를 폐포대식세포에 생체외로 투여한 후 활성산소종 생성을 chemiluminescence assay로 측정하였다.

결 과 : Silica-H^+, 또는 silica-Fe^{3+} 생체내 투여 군은 생리식염수 투여군에 비하여 폐손상과 호중구의 폐포내 유입, 폐포대식세포에 의한 NO 생성이 의미있게 증가되었으나, silica-H^+와 silica-Fe^{3+} 투여군간의 유의한 차이는 없었다. 또한 silica-H^+, 또는 silica-Fe^{3+}를 폐포대식세포에 생체외로 투여한 경우 활성산소종의 생성은 의미있게 증가되었으나, 이들 silica투여군간의 유의한 차이는 없었다.

결 론 : Fe^{3+}가 결합된 유리규산은 생체내 또는 외 투여로 인한 폐 손상 및 염증 반응에 영향을 끼치지 않는다.

중심 단어 : 표면철이 결합된 유리규산 · 폐염증.

Introduction

Pulmonary deposition of crystalline silica can result in a cycle of lung damage, fibroblast proliferation and excess collagen production in the lung, causing lung fibrosis or silicosis[1]. Upon contact with silica, alveolar macrophages produce a variety of inflammatory and fibrogenic factors, such as reactive oxygen species(ROS), lipid mediators, cytokines(IL-1, IL-6, and TNFα), chemokines, and macrophage-derived growth factors[2][3], which are critical to silica-induced pathogenesis.

In an aqueous environment, silica(SiO_2) is covered

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with surface hydroxyl groups, and silica surface has some concentration of silanol groups(-Si-OH) to cordinate ferric iron[4][5]. The ferric ion(Fe^{3+}) complexed by the surface of this dust allows silica to function as Fenton catalysts, resulting in the production of OH by an electron exchange via the Fenton reactionen[6] :

$Fe^{3+} + e^- \leftrightarrow Fe^{2+}$ ----------(1)

$Fe^{2+} + H_2O_2 \leftrightarrow Fe^{3+} \cdot OH + OH^-$ ----------(2)

The ability of silica to generate oxidants via the Fenton reaction has been established in vitro, indicating a role for iron in ROS generation by silica[7]. A role for iron in vivo generation of ROS by silica has also been demonstrated[8]. Therefore, complexation of inorganic and body sources of iron on silica surfaces may result in oxidant generation[9].

Recent evidence suggests that alveolar macrophage- and neutrophil-derived nitrogen free radicals may play important roles in mediative cytotoxicity through damping DNA and inhibiting the mitochondrial respiratory cycle[10][11]. In the previous study, it was found that inducible nitric oxide synthase(iNOS) mRNA expression and nitric oxide production by alveolar macrophages and recruited neutrophils were increased in acute inflammatory lung injury following exposure to silica. Nitric oxide(NO) may also combine with superoxide anion(oxidant generated via the Fenton reaction) to produce an even more cytotoxic molecule, peroxinitrite($ONOO^-$)[12]. Peroxinitrite is a long-lived, reactive oxidant that contributes to inflammatory tissue damage. In addition, cytotoxic effects of NO can be explained by its interaction with iron. It has been demonstrated that the loss of activity of the critical Krebs-cycle enzyme aconitase in activated macrophages is a result of the formation of iron-nitrosyl complexes[13][14].

The present study examines whether surface complexed Fe^{3+} influences silica-induced pulmonary responses. To address this objective, we (1) assessed the degree of lung injury and neutrophil transmigration into the alveolar spaces by determining the protein amount, and by measuring neutrophil counts with differential analysis in bronchoalveolar lavage fluid and measured nitric oxide levels in alveolar macrophage culture in animals in vivo exposed to silica complexed with H^+ or Fe^{3+} ; (2) measured reactive oxygen sepceies in alveolar macrophages in vitro exposed to these cation-complexed silica.

Materials and Methods

1. Preparation of surface complexed silica

The cation-complexed silica dusts(silica complexed with H^+(silica-H), and Fe^{3+}(silica-Fe^{3+})) were prepared by Dr. A.J.Ghio(Duke University, U.S.A.) and delivered for this study. In the present study, the relationship between the ability in the transport of electrons of these surface cations and silica-induced pulmonary responses was examined. Briefly, silica(2mg/ml) was stirred in solutions of either 1 mM HCl, or $FeCl_3$ for 15 min to provide each cation-loaded silica, respectively. They were then centrifuged at 1,200g for 10 min and washed 10 times in water. Silica was then dried at 37℃ for 4 days. In addition, Ghio et al[5]. Determined the ability of silica to further complex Fe^{3+} from solution of 1.0mM $FeCl_3$ where silica was found to have significantly elevated cheatable iron concentration of $61.3 \pm 1.5 \mu M/g$.

2. Cellular isolation

The rats were anesthetized with 0.2g/kg body weight of sodium pentobarbital. The trachea was then cannulated and the lungs were lavaged 10 times with 8ml aliquots of Ca^{++}, Mg^{++}-free Hanks balanced salt solution(145mM NaCl, 5mM KCL, 1.9mM NaH_2PO_4 and 5.5mM dextrose ; Ph=7.4). The bronchoalveolar lavagate was centrifuged(500g for 5 min), and the bronchoalveolar lavage cells were washed and resuspended in Hepes-buffered solution(145mM NaCl, 5mM KCL, 10mM Hepes, 5.5mM dextrose and 1.0mM $CaCl_2$; pH=7.4). Cell counts and differentials were determined using an electronic coulter counter with a cell sizing analyzer(Coulter Model ZBI with a channelizer 256 ; Coulter Electronics, FL, USA), as described by Lane & Mehta[15]. Red blood cells(RBC),

lymphocytes, polymorphonuclear neutrophils, and alveolar macrophage were distinguished by their characteristic cell volumes[16].

3. In vivo treatment with silica

The experimental design consisted of three groups of rats(specific pathogen-free male Sprague-Dawley rats weighing 260-290g) : (1) a saline-treated group that received an intratracheal(IT) instillation of 0.5ml of LPS-free saline(0.9% NaCl) ; (2) a silica-H^+- ; and (3) a silica-Fe^{3+}-treated group which received IT instillation of 5mg silica complexed either with H^+ or Fe^{3+} in 0.5ml saline, respectively.

Rats were anesthetized with intraperitoneal injection of 0.5ml of a 1% solution of sodium methohexital. After that, they were intratracheally instilled with saline, silica-H^+ or silica- Fe^{3+} using 20-gauge, 4-inch ball-tip animal feeding needle[17].

4. Measurement of total protein in BAL fluid after in vivo treatment with silica

Total proteins were measured in the first aliquot of the acellular bronchoalveolar lavage fluid. Using bovine serum albumin as a standard, the measurement of total protein was performed according to the method of Hartreea[18] to assess the permeability of the bronchoalveolar-capillary barrier.

5. Measurement of nitrate in alveolar macrophage culture after in vivo treatment with silica

Bronchoalveolar lavage cells were suspended in MEM(medium essential medium) with 10% fetal bovine serum, 2mM glutamine, 100u/ml penicillin, and 100g/ml streptomycin at a final concentration of 1×10^6 alveolar macrophages/ml. From cell suspension, 1ml aliquots were added to 24 well plates(Castar, MA, USA) and incubated for 2 h at 37℃ in a humidified atmosphere of 5% CO_2. The nonadherent cells were then removed with two 1ml washes of the fresh MEM. The adherent cells were counted microscopically and found to consist of >95% alveolar macrophages. After incubating for 24 h, the cell cultures were centrifuged at 500g for 15 min and the supernates frozen at -70℃ untill they were assayed.

To measure total nitrate(nitrite plus nitrate), nitrate in the samples was first reduced to nitrite with nitrate reductase, and the nitrite reaction was then determined with Greiss reagent. Nitrite was assayed after adding 100μl Greiss reagent(1% sulfanilamide and 0.1% naphthylethylenediamide in 5% phosphoric acid) to 50μl samples of cell culture. Optional density at 550nm(OD_{550}) was measured using a microplate reader. Nitrite concentrations were calculated by comparison with OD_{550} of standard solutions of sodium nitrite prepared in cell culture medium and presented as nmole/mg protein.

6. Measurement of chemiluminescence in alveolar macrophages after in vitro treatment with silica

The ability of silica to produce reactive oxygen species(ROS) by macrophages(RAW264.7 cells) was determined by measuring cellular chemiluminescence using a luminometer(Berthold, Model : LB9505AT, Wildbad, West Germany). Briefly, cells were washed once with phosphate-buffered saline(145mM NaCl, 5mM KCl, 1.9mM NaH_2PO_4, 9.35mM Na_2HPO_4, 5.5mM glucose, Ph 7.4), centrifuged and resuspended in HEPES-buffered medium(145mM NaCl, 5mM KCl, 10mM NaHEPES, 5.5mM glucose, 1mM $CaCl_2$, pH 7.4).

Alveolar macrophages(110^6 cells/ml) were preincubated for 10 min at 37℃ in a shaking bath and then stimulated with Si-H^+ or Si-Fe^{3+}(0.5-1.5mg/ml). Chemiluminescence was monitored continuously at 37℃ for 10 min in the presence of 8μg% luminol. The integral of cpm versus time was used to compare the total CL between samples.

7. Statistic analysis

Values were expressed as means standard error of means(SEM), and compared among experimental groups by one-way ANOVA. Significance was set at $p\leq 0.05$.

Results

To evaluate and compare acute inflammatory lung

injury in the rats exposed to silica complexed with H^+ or Fe^{3+}, neutrophil numbers and protein contents in bronchoalveolar lavage fluids were analysed. Compared with the saline group, the number of neutrophils was significantly increased following IT silica-H^+ or -Fe^{3+}($p \leq 0.05$, Fig. 1). However, there was no significant difference in neutrophils between the rats treated with these cation-complexed silica($p \leq 0.05$).

As shown in Fig. 2 protein contents in the lavage fluid were significantly increased by about 3-4 fold in silica complexed with H^+ or Fe^{3+} compared with the saline group($p \leq 0.05$), whereas there was no significant change between these silica-treated groups($p < 0.05$).

Increasing evidences suggest that a large part of silica-induced alveolar damage is due to the production of alveolar macrophage-derived cytotoxic species such as NO. Cytotoxic effects of NO can be explained by its interaction with iron. Therefore, the relationship between surface iron complexation and the ability of silica to induce NO production by alveolar macrophages was examined.

Nitrate, an oxidation end product of nitric oxide, was measured in alveolar macrophages cultured for 24 h after exposure to silica-H^+ or Fe^{3+}. Treatment of any one of the complexed silica increased nitrate by 2 fold above the levels of saline controls($p \leq 0.05$) (Fig. 3). However, Fe^{3+} complexation onto the silica surface did not enhance silica ability to stimulate NO production by alveolar macrophages.

In vitro treatment of alveolar macrophages with Si-H^+ or Si-Fe^{3+}(0.5 – 1.5mg/ml) resulted dose dependent increase in ROS production, measured as the generation of chemiluminescence with a maximal enhancement of 1.9 or 1.4 fold at 1.0mg/ml these cation-complexed silica, respectively(Fig. 4). However, there was no significant difference in cellular chemiluminescence at each concentration of Si-H^+ or Si-Fe^{3+}.

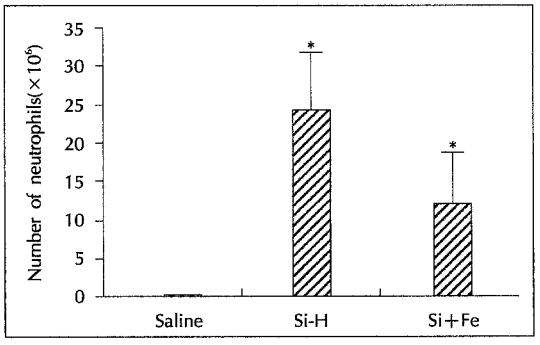

Fig. 1. Neutrophil in bronchoalveolar lavage cells. 4 h after it instillation with saline, silica-H^+ or Fe^{3+}(5mg in 0.5ml saline), bronchoalveolar lavage cells were counted and differentiated using an electronic coulter counter with a cell sizing analyzer. Values represent mean±SEM from 4 experiments. *significant difference compared with control(IT saline instilled), $p \leq 0.05$.

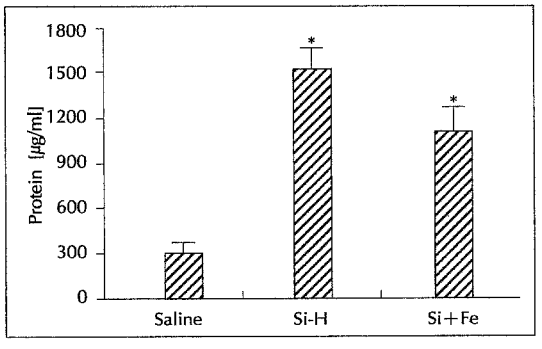

Fig. 2. Total protein content in bronchoalveolar lavage fluid. Rats were it instilled with saline, silica-H^+ or Fe^{3+}(5mg in 0.5ml saline). After 4 h, protein content in the bronchoalveolar lavage fluid was measured spectrometrically at 560nm. Values represent mean±SEM from 4 experiments. *significant difference compared with control(IT saline instilled), $p \leq 0.05$.

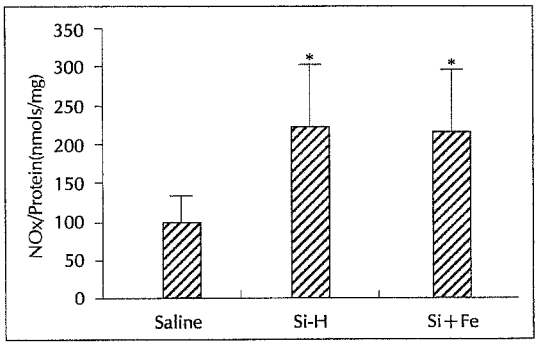

Fig. 3. Nitric oxide(NO) production in the cultured alveolar macrophages. 4 h after it instillation with saline, silica-H^+ or Fe^{3+}(5mg in 0.5ml saline), were cultured for 24 h and no production was measured using nitrate assay. Values represent mean±SEM from 4 experiments. *significant difference compared with control(IT saline instilled), $p \leq 0.05$.

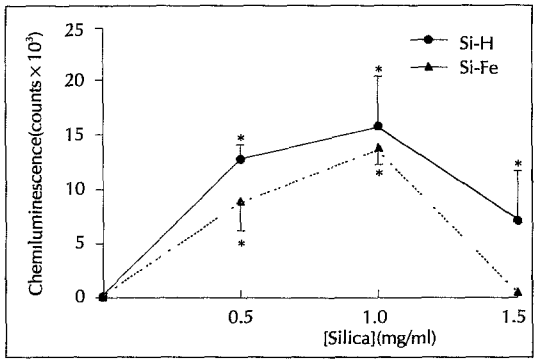

Fig. 4. Generation of chemiluminescence from alveolar macrophages after in vitro treatment with silica-H^+ or Fe^{3+}. The cells(110^6/ml) were preincubated at 37℃ for 10 min and then stimulated with silica silica-H^+ or Fe^{3+}(5mg in 0.5ml saline). Silica-stimulated chemiluminescence0 was expressed as the integral of cpm versus time. Values represent mean± SEM from 4 experiments. *significant difference compared with control(IT saline instilled), $p \leq 0.05$.

Discussion

The results show that intratracheal instillation of each of silica complexed with H^+ or Fe^{3+} caused increases in the nhumber of neutrophils and total protein contents in bronchoalveolar lavage fluid. However, compared with the rats exposed to H^+- complexed silica, Fe^{3+} complexation onto the silica surface did not significantly change the number of neutrophils and total protein contents in the lavage fluid. These data indicate that the ability of silica itself to induce damage to the lung parenchyma and the alveolar-capillary barrier was not influenced by surface complexed with Fe^{3+}. In contrast to these findings, Ghio et al[5] have demonstrated silica complexed with Fe^{3+} greatly increased both the cellular influx, including the number of neutrophils, and protein contents in the larvage fluid obtained from the rats 96 h after treatment with wetted silica. This disagreement may be due to the time exposed to silica, i.e., 4 h in the present study vs 96 h in the study by Ghio et al[5]. Recently, Ghio et al[19] have characterized changes in the concentration of ferritin and lactoferrin in the lower respiratory tract after exposure of rats to an emission source air pollution particle. The highest concentration of these iron-binding proteins occurred at approximately 24 h after exposure to the air pollution particle, and then the concentration started to decrease. By 96 h after instillation, the concentration was not elevated compared to the animals exposed to saline. Therefore, 4 h of exposure as performed in the present study rather than 96 h may be more appropriate to determine whether surface complexed metal is responsible for silica-induced pulmonary inflammation because when the exposure time delayed over 96 h, more intricated factors reflecting the host environment such as kinetics of the concentration of iron-binding proteins may be involved in the experimental system.

NO production by alveolar macrophages from the rats treated with these cation-complexed silica was also significantly increased compared with the saline-treated rats. However, NO production was not significant between the rats treated with silica-H^+ and silica-Fe^{3+}.

In vitro treatment of alveolar macrophages with silica also results in a significant increase in ROS production while there was no significant differences between silica-H^+ or silica-Fe^{3+}. These results of NO and ROS production are supported by the findings of Kamp et al[8], where iron was not required to generate the reactive radical, suggesting silica also appears to catalyze electron transfer reactions which do not require iron.

It is concluded that silica surface complexed with Fe^{3+} does not influence acute lung inflammation and NO production by alveolar macrophages after 4 h in vivo treatment and ROS production by alveolar macrophages after in vitro treatment.

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