Expression of Heme Oxygenase-1 in the Lungs of Rats Exposed to Potassium Octatitanate Whiskers

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Abstract: Expression of Heme Oxygenase-1 in the Lungs of Rats Exposed to Potassium Octatitanate Whiskers: Yasuko Obata, et al. Institute of Industrial Ecological Sciences, University of Occupational and Environmental Health, Japan—Objectives: Oxidative stress is thought to be the pathogenesis of pulmonary fibrosis induced by asbestos, and heme oxygenase-1 (HO-1) protects lung tissue against oxidative stress. We hypothesized that HO-1 is also associated with oxidative lung injury caused by exposure to potassium octatitanate whiskers (PT1), which is one of the asbestos substitutes. Methods: Male Wistar rats were administered 1 mg or 2 mg PT1 suspended in saline by a single intratracheal instillation and were sacrificed after recovery for 3 days, 1 wk, 1 mo, 3 mo or 6 mo. Gene expression of HO-1 protein and mRNA and immunostaining were investigated in rat lungs. Results: HO-1 protein expression was increased from 3 days to 1 mo and at 6 mo in the 1 or 2 mg PT1-exposed groups, and the gene expression of HO-1 mRNA was also increased at 3 days and from 1 mo to 6 mo. HO-1-positive cells were mainly found in the alveolar macrophages and the bronchial epithelial cells in immunostaining. Conclusions: These findings suggest that HO-1 is involved in lung damage caused by PT1. (J Occup Health 2011; 53: 267–273)

Key words: Heme oxygenase-1 (HO-1), Intratracheal instillation, Oxidative stress, Potassium octatitanate whisker (PT1)

It is widely accepted that exposure to asbestos causes lung injury including lung fibrosis, lung cancer and malignant mesothelioma. Accordingly, man-made mineral fibers are being promoted and used as a substitute for asbestos, and their demand is substantially increasing. Potassium octatitanate whisker (PT1) is one of these man-made fibers and asbestos substitutes with a high resistance to heat, friction and chemicals, and it also has a high mechanical strength. It has been widely used in various applications including brake pads, construction materials of outer walls requiring good resistance to weather, coating materials with extra weatherability, protective coverings for cultural properties and antidecoloring coating agents. PT1 is composed of 100% K₂O • 8TiO₂ and has a crystalline structure, low solubility and high retention in the lung. Since lung tumor has been observed in a long-term inhalation study in rats, there has been concern about potential effects on human health.

Exposure to dust such as asbestos dust could cause lung injury that initiates certain responses including inflammation due to oxidative stress mediated by oxygen radicals and desquamation of the alveolar lining. As repair processes are initiated, fibrogenic and carcinogenic changes in the lung ensue.

Since heme oxygenase-1 (HO-1) is known to protect lung tissue against various kinds of oxidative stress, increased expression of HO-1 is thought to reflect oxidative stress. Expression of HO-1 is significantly induced by oxidative stress such as ultraviolet rays, heat shock, endotoxin and cytokine. In the heme degradation process, HO-1 acts as a rate limiting enzyme and produces bilirubin, ferritin and carbon monoxide (CO), and the protective system is mediated by the physiologic activities of these products. Increased HO-1 expression has also been reported in human interstitial pneumonia (UIP) and cystic fibrosis (CF). Increased HO-1 expression has also been found in our study of a lung injury model in which...
rats were exposed to asbestos and crystalline silica; therefore, HO-1 is thought to be related to lung injury caused by dust.

In this study, we investigated the involvement of HO-1 gene expression in lung injury caused by PT1, a man-made mineral fiber being used as an asbestos substitute, using a model in which animals were exposed to PT1.

**Materials and Methods**

**Animals**

The male Wistar rats (10 wk old) used in this study were purchased from Kyudo (Kumamoto, Japan). Either saline or PT1 suspension (1 mg or 2 mg/0.4 ml saline) was administered to the animals intratracheally. The animals were allowed to recover for 3 days, 1 wk, 1 mo, 3 mo and 6 mo. They were assigned to an exposed group and control group for each exposure category, and each group contained 5 rats. After completion of the exposure time, the animals were sacrificed with an overdose of phenobarbital by intraperitoneal injection. The rats were handled according to the guidelines described in the Japanese Guide for the Care and Use of Laboratory Animals as approved by the Animal Care and Use Committee, University of Occupational and Environmental Health, Japan.

**Particles**

The PT1 used in this study was the JFM standard reference sample from the Japan Fibrous Materials Research Association (JFMRA). It was measured using scanning electron microscopy. It had a geometric mean length of 4.4 µm (geometric standard deviation, GSD 2.7) and a geometric average diameter of 0.35 µm (GSD 1.6; Fig. 1).

**Expression of HO-1 protein**

Lung tissue proteins were extracted from the right lung. Samples were prepared by homogenization of rat lungs that were treated with saline and PT1. Protein concentrations of lung homogenates were adjusted to 2000 µg/ml, and then expression of HO-1 protein was studied by an ELISA method using a Rat HO-1 ELISA Kit (Stressgen Bioreagents, Ann Arbor, MI, USA).

**Gene expression of HO-1 mRNA**

RNA was extracted from the right lung, and single-strand cDNA was synthesized using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Forster, CA, USA).

Real-time polymerase chain reaction (PCR) and subsequent calculations were performed with a 7500 Real-Time PCR System (Applied Biosystems, Foster city, CA, USA), which detects the signal emitted from fluorogenic probes during PCR. Primers and probes were designed according to guidelines from Applied Biosystems with the Primer Express 3.0 software (Applied Biosystems, Foster city, CA, USA). The specificity of the nucleotide sequences chosen was confirmed by BLAST searches. The primer sets were as follows: heme oxygenase 1, Assay ID Rn00561387 and for β-actin (Rat ACTB) Lot No, 0710010.

Real-time PCR was performed with TaqMan Universal PCR Master Mix reagents. The PCR mixture contained 25 µl of TaqMan Universal PCR Master Mix (2x), 2.5 µl of TaqMan Gene Expression Assays and 17.5 µl of d-water in a total volume of 45 µl. PCR was performed using 5 µl of the first strand cDNA mix. After 2 min at 50°C to permit UNG cleavage, AmpliTaq Gold was activated by a 10 min incubation at 95°C. Each of the 50 PCR cycles consisted of a 15-sec denaturation step at 95°C and a hybridization step, with probes and primers and for DNA synthesis, for 1 min at 60°C.

The average cycle threshold (C_T) was determined for each group of animals at each time point. Relative gene expression was calculated using the comparative C_T method, which assesses the difference in gene expression (ΔC_T: difference between the threshold cycle) between the HO-1 gene and β-actin for each sample to generate the ΔΔC_T. Relative gene expression was then determined by the formula 2^(-ΔΔCT). The average relative expression in each group was calculated with respect to the control group in each time point, and the relative expression of the control group was set as 1.

**Expression of nitrite and nitrate protein**

The final products of nitric oxide (NO) in vivo are nitrite and nitrate. The relative proportion of nitrite and nitrate is variable and cannot be predicted with certainty. Thus, the best index of total NO production is the sum of both nitrite and nitrate. The protein concentrations in the lung...
homogenates were adjusted to 4,000 µg/ml, and then nitrate/nitrite levels were determined by the ELISA method using a Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical Company, Ann Arbor, MI, USA).

Tissue preparation for immunohistochemistry
After removal of the right lung, the left lung was inflated and fixed by intratracheal instillation of 4% paraformaldehyde at a pressure of 25 cm H2O. The lung and trachea were resected from the surrounding tissue and allowed to stand at 4°C for 24 h. The tissue was washed for 10 min in phosphate-buffered saline, dehydrated by immersion in a graded series of ethanol washes for 1 h per wash and then maintained in 100% ethanol at 4°C. The lung tissue was embedded in paraffin, and sections were cut out from the lobe. Immunostaining for HO-1 was performed using 3-µm sections. The slides were placed in methanol containing 0.3% hydrogen peroxide to block endogenous peroxidase activity. To minimize background staining, the sections were blocked with 10% normal goat serum. We incubated the lung sections overnight at 4°C with rabbit anti-rat HO-1 antibody from Stressgen (Victoria, BC, Canada). The sections were rinsed three times in 0.1 M phosphate-buffered saline (PBS) and incubated for 30 min at room temperature with an anti-rabbit secondary antibody (Histofine Simple Stain Rat MAX-PO (R), Nichirei, Japan). The sections were washed three times, incubated with diaminobenzidine and counterstained with hematoxylin. Incubations were carried out without the primary or secondary antibody as a labeling control.

Statistical analysis
We used the non-parametric Mann-Whitney statistical test for the PT1-exposed and control groups. Values were expressed as the mean ± one standard deviation of 5 animals. Differences at $p<0.01$ and $p<0.05$ were considered statistically significant by the test.

Results

Expression of HO-1 protein (Fig. 2)
The HO-1 levels in the lung tissue of the PT1-exposed groups were significantly increased from 3 days to 1 mo after exposure, but increased (though not significantly) at 3 mo and increased significantly at 6 mo compared with the control groups. The HO-1 levels of the 1-mg PT1-exposed groups were increased at 3 days and 1 wk after exposure to a lesser extent than the 2-mg PT1-exposed groups, and the levels were not significant but increased from 1 mo to 6 mo.

Gene expression of HO-1 mRNA (Fig. 3)
Increased expression of HO-1 mRNA in both the 1-mg and 2-mg PT1-exposed groups was observed during the acute and chronic phases. Significant increases in gene expression of HO-1 in the PT1-exposed groups were observed at 3 days and from 1 mo to 6 mo after exposure compared with the control groups.

Expression of nitrite and nitrate (Fig. 4)
No significant increase of NO was observed in the 1-mg and 2-mg PT1-exposed groups compared with the control groups.

Histopathological findings (Fig. 5)
Infiltration of inflammatory cells (mainly neutrophils and alveolar macrophages) was partly observed in the 2-mg PT1-exposed groups at 3 days after exposure, but
this tended to fade away at 6 mo.

**Immunohistochemical staining for HO-1 gene (Fig. 6)**

HO-1-positive cells were found in alveolar macrophages, mainly in phagocytosed PT1 fibers, and some positive cells were found in the alveolar and bronchial epithelial cells.

There were markedly more positive cells, and many fibers phagocytosed by macrophages were found in the lungs at 3 days after exposure. It was our impression that there were markedly more HO-1-positive macrophages up to 3 mo, that they decreased at 6 mo and that there were markedly more in the bronchial epithelial cells at 6 mo.

**Discussion**

The HO-1 protein levels in the lung tissue of the PT1-exposed rats were increased not only in the acute phase but also in the chronic phase. Nagatomo et al. examined
Fig. 6. Lung sections from PT1-exposed rats immunostained for HO-1. (A) Saline-exposed (control) lung after 3 days of recovery. Magnification ×400. (B) 2-mg PT1-exposed lung after 3 days of recovery. Magnification ×100. (C) 2-mg PT1-exposed lung after 3 days of recovery. HO-1 staining of the alveolar macrophages (open arrow) was more intense than that of the control group. Magnification ×400. (D) 2-mg PT1-exposed lung after 6 mo of recovery. Many bronchial epithelial cells (black arrow) show positive HO-1 staining. Magnification ×400.

HO-1 gene expression in animal lung tissue from 3 days to 3 mo after intratracheal instillation of crocidolite and reported a persistent increase in HO-1 gene protein expression. Similar intratracheal instillations of crystalline silica, fine silica and chrysotile showed increased HO-1 gene protein expression in both the acute and chronic phases. These findings reveal that asbestos and crystalline silica, which are hazardous to the lung, significantly and persistently increased the HO-1 protein expression. On the other hand, less hazardous titanium dioxide (TiO₂) did not induce an increased HO-1 expression.

The protein expression pattern of the HO-1 gene in the present study was persistent though mild in the chronic phase. It was not constant as in the case of crocidolite and crystalline silica and was different from transient expression in the acute phase as in the case of TiO₂. It was intermediate between these expression levels.

The pattern of HO-1 expression induced by PT1 is similar to the persistent pattern of pulmonary inflammation and fibrosis. We studied rat pulmonary inflammation and fibrosis by intratracheal instillation of known hazardous materials and PT1. Pulmonary inflammation was increased and lasted from the acute phase to the chronic phase in the study using hazardous silica and crocidolite; however, transient inflammation in the acute phase was found in less-hazardous TiO₂. As for PT1, inflammation was observed in the acute phase, and it lasted but tended to be decreased in the chronic phase. The persistency of pulmonary inflammation by PT1 tended to be intermediate between those of TiO₂ and crocidolite. These findings suggest that HO-1 expression, along with pulmonary inflammation, reflects a hazardous effect.

The protein production and mRNA expression of the HO-1 gene showed a similar tendency in terms of the increase from the acute phase to the chronic phase. There
was also a similar tendency in HO-1 protein expression and immunohistochemical staining for the HO-1 gene, with the expression being increased in the acute phase and decreased in the chronic phase. However, the protein expression of HO-1 was proceeded by mRNA expression of HO-1. Nagatomo et al. reported that the mRNA expression of HO-1 did not change in the early phase and was increased in the chronic phase in the chrysotile-exposed model, while it was slightly decreased in the early phase and mildly increased in the chronic phase in the animal model for TiO₂. Nagatomo et al. also reported an increased HO-1 gene expression and protein expression preceding mRNA expression in the chrysotile-exposed model; their findings were similar to the HO-1 gene expression pattern by PT1 in the present study.

The mechanism explaining this discrepancy may depend on increased HO-1-positive macrophages. Immunohistochemical staining showed that there were markedly more HO-1-positive alveolar macrophages in the acute phase and a decreased amount in the chronic phase in the present study. These results suggest that macrophages positive for HO-1 protein may have been induced and flowed into alveolar spaces. Therefore, the expression of protein (concentration of HO-1 protein / protein exactly) was increased from the acute phase as an acute inflammatory reaction. We have also reported that more alveolar macrophages were stained for HO-1 in rats exposed to crocidolite, chrysotile and crystalline silica compared with the control groups. Maestrelli et al. reported that HO-1-positive macrophages were increased in smokers with chronic obstructive pulmonary disease (COPD) as compared with nonsmoking subjects in an immunohistochemistry study of human lung surgical specimens.

Many transcription factors concerned with activation of the HO-1 gene are known, including heat shock factor (HSF), nuclear factor-kappa B (NF-kB), nuclear factor-erythroid 2 (NF-E2), NF-E2-related factor 2 (Nrf2) and activator protein-1 (AP-1) (4). The transcription factors for NF-kB and mitogen-activated protein kinase (MAPK) signaling pathways are assumed to be activated when HO-1 expression increases. A study of the fibrosis model induced by bleomycin and a study of human macrophages exposed to chrysotile reported activation of NF-kB. No studies have reported the increased expression of NF-kB after PT1 exposure; however, a study reported tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and transforming growth factor-β1 (TGF-β1) expression, which were activated by NF-kB after PT1 inhalation exposure (5).

A previous study reported that HO-1 was thought to be induced by reactive oxygen species (ROS) and reactive nitrogen species (RNS) as an antioxidative defense factor. The present study did not observe an increase of NO production by PT1; thus, the increase in HO-1 may be induced by ROS. Another report described that HO-1 expression was induced by an exposure to ozone, one type of ROS, in a mouse study. TGF-β1 activation is induced by exposure of A549 cells to chrysotile and crocidolite, and a study reported that ROS is involved in that activation (6).

Motterlini et al. reported that NO was a determinant when heme oxygenase induced major damage of the epithelium under oxidative stress; however, the present study showed no NO-related expression throughout the observation period. Tanaka et al. observed an increase of nitrotyrosine in the rat lung by ELISA during an observation period of 1–6 wk after inhalation of crocidolite and chrysotile and reported that nitric oxide synthase activity and peroxynitrite formation were induced by asbestos inhalation. The present study did not show a marked increase of nitrite and nitrate and suggests that the damage by NO caused by PT1 is less severe than that of crocidolite and chrysotile.

In conclusion, HO-1 gene expression in the rat lung was examined by intratracheal instillation of PT1. HO-1 gene expression was increased mildly and persistently throughout the observation period, and the severity of pulmonary inflammation was intermediate between that of the positive controls, such as silica and crocidolite, and negative controls, such as TiO₂. Therefore, HO-1 is involved in lung damage caused by PT1.

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References
6) Zhou H, Lu F, Latham C, Zander DS, Visner GA. Heme oxygenase-1 expression in human lungs with cystic fibrosis and cytoprotective effects against Pseudomonas


