Antioxidant Activities in the Lung of Murine Hermansky-Pudlak Syndrome (HPS) Model: Effect of Ionizing Radiation

Ho-Sang Shin, Woo-Jung Yang[1] and Eun-Mi Choi[*]

Department of Chemistry, University of Incheon,
177 Dohwadong, Namgu, Incheon 402-749, Korea
[1]*Asia Pharmaceuticals, Asia B/D, 413-13 Shindolimdong, Gurogu,*
Seoul 152-887, Korea

Hermansky-Pudlak Syndrome (HPS) 생쥐 모델의 폐 항산화계 활성: 방사선의 영향

신 호 상, 양 우 정[1], 최 은 미[*]

인천대학교 화학과, [1]아주약품

요 약

Hermansky-Pudlak Syndrome (HPS) 환자에서 조기에 발생되는 폐섬유화의 원인을 알아보고자, 생쥐 HPS 모델인 *ep/ep,pe/pe* 돌연변이종의 폐 항산화계의 활성과 방사선에 대한 반응을 측정하였다. HPS 폐에 서는 대조군에 비해 glutathione이 더 산화되어 있었고, catalase, glutathione S-transferase (GST) 등의 항산 화효소의 활성이 저하되어 있었으며, 10 Gy의 방사선을 조사하였을 때, glutathione 양이 감소하였고, 대조 군 폐에서 보여 지는 방사선에 의한 γ-glutamylcysteine ligase (GCL), glutathione peroxidase (GPx) 활성의 유의성 있는 증가가 관찰되지 않았다. 이 결과로부터 HPS 환자의 폐는 항산화계 활성이 저하되어 있을 뿐 아니라, 산화적 스트레스가 가해 졌을 때 적응 반응이 매우 취약하여 산화적 환경에 노출된 폐의 병증 을 유발할 수 있음을 추측할 수 있다.

Key words : Hermansky-Pudlak syndrome, antioxidants, radiation, oxidative stress

INTRODUCTION

Hermansky-Pudlak Syndrome (HPS) is an autosomal recessive disorder characterized by oculocutaneous albinism, bleeding tendency, and ceroid depo-

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※ To whom correspondence should be addressed.
Tel: +82-32-770-8233, Fax: +82-32-770-8238
E-mail: eunmi@incheon.ac.kr

sits in the reticuloendothelial system (Hermansky and Pudlak, 1959; Witkop *et al.*, 1990). The most serious clinical problem associated with HPS is lung fibrosis, which may lead to death in midlife (Brantly *et al.*, 2000; Nakatani *et al.*, 2000; Huizing *et al.*, 2001). HPS lung disease presently has no cure, and its molecular cause (s) is unknown. The incidence and severity of pulmonary insufficiency varies widely among HPS patients.

Six genetically distinct forms of HPS have been described in humans (Lyerla *et al.*, 2003). It has been known that the HPS genes are involved in the bio-genesis and trafficking of lysosome-related organelles such as melanosomes, dense granules of platelet, and lysosomes (Spritz, 1999; Anikster *et al.*, 2001). HPS1 is most prevalent and is caused by mutations in the HPS1 gene (Oh *et al.*, 1996). HPS2 (AP3B1) is caused by mutations in the β_{3A}-subunit of the adaptor protein-3 (AP-3) adaptor complex, which is well known to regulate endocytotic vesicle trafficking (Dell'Angelica *et al.*, 1999; Robinson and Bonifaci-no, 2001). The remaining HPS forms (HPS3, HPS4, HPS5, and HPS6) arise from mutations in novel vesi-cle-trafficking genes (Anikster *et al.*, 2001; Suzuki *et al.*, 2002; Zhang *et al.*, 2003). The incidence of the lung disease among HPS patients appears greatest in HPS1 and HPS4, although there are relatively few relevant studies in the other genetic forms of HPS (Brantly *et al.*, 2000; Avila *et al.*, 2002).

At least 16 mouse hypopigmentation mutants accu-rately model HPS (Swank *et al.*, 1998; Swank *et al.*, 2000; Zhang *et al.*, 2003). Several of these mouse HPS model strains bear mutations on orthologues of the human HPS genes. The corresponding mouse models for the various forms of human HPS contain genes that encode proteins involved in vesicle traffick-ing with either well-established or unknown roles. There are a limited number of studies of lung abnor-malities in mouse HPS mutants. Mutants homozy-gous for both the pale ear (*Hps1* or *ep*) and pearl (*Ap3b1* or *pe*) HPS gene (*ep/ep,pe/pe* mutant), pro-duced in Dr. Richard T Swank's laboratory (Feng *et al.*, 2002) exhibit more severe mutant phenotype for all the common lysosome-related organelles (melano-somes, platelet-dense granules, and lysosomes), indi-cating that these genes cooperate in regulating differ-ent aspects of vesicle trafficking. Levels of lung lyso-somal enzymes are significantly elevated in the *ep/ep,pe/pe* mutant, suggesting possible abnormalities of lung lysosome-related organelles, such as lamellar bodies (Weaver *et al.*, 2002) of type II cells. Indeed, prominent morphological and biochemical abnormal-ities in lung type II cells of *ep/ep,pe/pe* mouse mu-tants, similar to those reported in type II cells of Her-mansky-Pudlak patients (Nakatani *et al.*, 2000), toge-ther with aberrant lung function, were reported (Lyerla *et al.*, 2003). The air spaces of the mutant lungs con-tain age -related elevations of inflammatory cells and foamy macrophages (Lyerla *et al.*, 2003). However, there is little experimental data for the underlying mechanisms for lung damage in HPS patients.

As an effort to probe for the early development of pulmonary functional abnormalities and fibrosis in HPS patients, we evaluated pulmonary antioxidant activities and their response to oxidative stress, ioniz-ing radiation, of murine HPS model, *ep/ep,pe/pe* mouse mutants, considering a role of oxidative stress in lung injuries ranging from increased inflammation to lung fibrosis associated with HPS. The antioxidant system we analyzed includes glutathione, a small molecular thiol antioxidant; γ-glutamylcysteine ligase (GCL) that is a rate-limiting enzyme for glutathione synthesis; glutathione peroxidase (GPx), glutathione reductase (GRd), glucose-6-phosphate dehydrogenase (G6PD) and glutathione S-transferase (GST) that are involved in glutathione recycling metabolism; and superoxide dismutase (SOD) and catalase that are reactive oxygen species (ROS) scavenging enzymes. Our results suggest a possible association of defective antioxidant system with pulmonary abnormality in HPS patients.

MATERIALS AND METHODS

1. Materials

Glutathione (reduced, GSH; oxidized, GSSG), 2, 4-dinitrofluorobenzene (FDNB), NADPH, NADP$^+$, NADH, phosphoenolpyruvate, glucose-6-phosphate, xanthine, 1-chloro 2, 4-dinitrobenzene (CDNB), GRd, pyruvate kinase (PK), lactate dehydrogenase (LDH), xanthine oxidase (XO), cytochrome *c* were purchased from Sigma (St. Louis, Mo, USA). Spheri-sorb NH$_2$ column (particle size 5 μm, 25 cm × 4.6 mm) was from ISCO (Lincoln, NE, USA).

2. Animals and irradiation

Male C57BL/6J mice and *ep/ep,pe/pe* double mutant mice, which are C57BL/6J inbred strain (8 ~ 10 weeks old) were generously provided by Dr. RT Swank at Roswell Park Cancer Institute. Whole body irradiation (single 10 Gy dose) was performed by using 4 MV photon beams (MeVatron, Siemens, Germany). Irradiation dose was calculated at the mid depth of mice in the field size of 40 cm with a dose rate of 0.2 Gy/min as previously described (Lee *et al.*, 2002). Mice were sacrificed and the lungs were removed 6 h or 24 h after irradiation. The tissues were washed and frozen by freeze-clamping with dry ice-cooled tongs immediately. Tissue samples were stored at $-80°C$ until analyzed.

3. Glutathione and enzyme activity analyses

Glutathione was measured by using HPLC as described previously (Park *et al.*, 1998). Briefly, 5% perchoric acid extract of the lung was derivatized with FDNB, and GSH and GSSG were separated by HPLC on a Spherisorb NH_2 column (Reed *et al.*, 1980). Total glutathione was expressed as GSH equivalent to the sum of GSH and GSSG, that is, $GSH+2$ GSSG. GCL activity was measured by monitoring oxidation of NADH at 340 nm in reaction mixtures containing 140 mM Tris-HCl (pH 8.2), 10 units/mL LDH, 10 units/mL PK, 75 mM KCl, 25 mM $MgCl_2$, 10 mM ATP, 5 mM L-glutamate, 10 mM α-amino-L-butyrate, 0.2 mM NADH, 0.2 mM EDTA, and 1 mM phosphoenolpyruvate (Seelig and Meister, 1984). GPx activity was measured by monitoring oxidation of NADPH at 340 nm in reaction mixtures containing 50 mM potassium phosphate (pH 7.4), 1 mM EDTA, 1 mM NaN_3, 0.2 mM NADPH, 1 unit/mL GRd, 1 mM GSH and 0.25 mM H_2O_2. The reaction mixture except H_2O_2 was incubated for 5 min, and the reaction was initiated by the addition of H_2O_2 (Paglia and Valentine, 1967). GRd activity was measured by monitoring oxidation of NADPH in reaction mixtures containing 100 mM potassium phosphate (pH 7.4), 2 mM GSSG, 0.6 mM EDTA, and 0.5 mM NADPH (Cohen

and Duvel, 1988). G6PD activity was measured by monitoring reduction of $NADP^+$ at 340 nm in reaction mixtures containing 50 mM Tris-HCl (pH 8.0), 50 mM $MgCl_2$, 2 mM $NADP^+$, and 4 mM glucose-6-phosphate (Bautista *et al.*, 1992). SOD activity was measured by monitoring reduction of cytochrome c at 550 nm in reaction mixtures containing 50 mM potassium phosphate (pH 7.5), 0.1 mM xanthine, 0.5 munit/mL XO, 0.1 mM EDTA and 10 µM cytochrome c. One unit of SOD represents the amount of enzyme that causes 50% inhibition in the reduction of cytochrome c (McCord and Fridovich, 1969). Catalase activity was measure by monitoring removal of H_2O_2 at 240 nm in reaction mixtures containing 50 mM potassium phosphate (pH 7.0) and 10 mM H_2O_2 (Aebi, 1974). GST activity was measured by measuring the rate of enzyme catalyzed the conjugation of GSH with CDNB spectrophotometrically at 340 nm in reaction mixtures containing 100 mM potassium phosphate (pH 6.5), 1 mM GSH and 1 mM CDNB (Habig *et al.*, 1974).

4. Statistical analysis

Data are expressed as means $\pm$ standard deviations. The significance of the differences between experimental and control groups was determined using the Student's t-test. *P* values of <0.05 were considered as significant.

RESULTS

1. Steady-state antioxidant capacity

We compared steady-state antioxidant capacity in the lungs of *ep/ep,pe/pe* mice (*ep/ep,pe/pe* lungs) with control C57BL/6J mice in order to determine whether *ep/ep,pe/pe* lungs are equipped with antioxidant capacity comparable to normal lungs. We analyzed the content of glutathione, the major small molecular antioxidant, and activities of various antioxidant enzymes (Table 1). Total glutathione content of *ep/ep,pe/pe* mice was similar to the control C57BL

Table 1. Comparison of steady-state antioxidant capacity between lungs of C57BL/6J and *ep/ep,pe/pe* mice

Antioxidant	C57BL/6J	*ep/ep,pe/pe*
Total glutathione (nmol/mg protein)	15.81±1.36	13.59±1.20
GSH/GSSG	9.97±1.29	7.04±0.64*
GCL (nmol/min · mg protein)	85.21±14.00	81.67±2.28
GPx (nmol/min · mg protein)	324.1±14.24	303.2±9.26
GRd (nmol/min · mg protein)	24.33±2.02	20.10±3.18
G6PD (nmol/min · mg protein)	48.93±6.50	52.16±5.07
GST (nmol/min · mg protein)	412.2±44.69	282.13±38.5*
SOD (unit/mg protein)	507.1±23.27	501.4±16.02
Catalase (μmol/min · mg protein)	31.62±2.20	24.06±2.67*

*$P < 0.05$

/6J mice, however, the ratio of GSH/GSSG was lower in *ep/ep,pe/pe* mice, indicating that the glutathione pool of *ep/ep,pe/pe* mice was more oxidized than the C57BL/6J mice. The antioxidant enzymes that we analyzed are first, glutathione metabolizing enzymes, which include GCL, GPx, GRd, G6PD and GST, and second, ROS scavenging enzymes, which include SOD and catalase. Our result showed that, among the various antioxidant enzymes we analyzed, GST and catalase activities were significantly lower in the *ep/ep,pe/pe* lung compared with the control C57BL/6J.

2. Radiation-induced changes in glutathione

We compared the ionizing radiation-induced changes in the antioxidant systems in the lungs of *ep/ep,pe/pe* mice with those of control C57BL/6J mice in order to determine the response of the *ep/ep, pe/pe* lungs to the oxidative stress caused by ionizing radiation. First, we analyzed the changes in glutathione content upon irradiation with 10 Gy (Fig. 1). Glutathione is not only the important antioxidant molecule, but the cellular content of glutathione is recognized as a sensitive indicator of oxidative stress, because glutathione is oxidized by enzymatic and nonenzymatic reactions with ROS, and subsequently
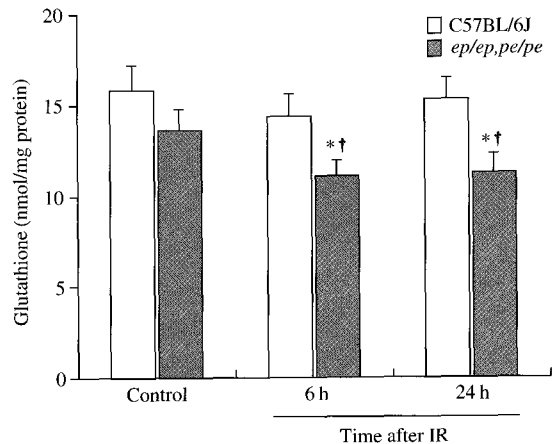

Fig. 1. Changes in total glutathione in lungs of C57BL/6J and *ep/ep,pe/pe* mice after total body irradiation. Mice were irradiated at a single dose of 10 Gy, sacrificed, and lungs were removed 6 h, or 24 h after irradiation. The acid extracts of the tissues were derivatized with FDNB, and glutathione content was analyzed by the HPLC method. Total glutathione was expressed as the GSH equivalent to the sum of GSH and GSSG. Results are expressed as means±standard deviations (n=5). *$P < 0.05$ vs. control C57BL/6J mice. †$P < 0.05$ vs. respective un-irradiated control.

depleted from cells under the oxidative stress. Basal level of the total glutathione did not show significant difference between the two. Irradiation with 10 Gy caused a decrease in glutathione content in the *ep/ep, pe/pe* lung within 6 h after irradiation, and the glutathione content did not recover for at least 24 h following irradiation. On the other hand, the glutathione content in the control C57BL/6J lung did not show significant decrease.

3. Radiation-induced changes in antioxidant enzyme activities

Then, we compared ionizing radiation-induced changes in antioxidant enzymes, that is, GCL (Fig. 2), Gpx (Fig. 3), GRd (Fig. 4), GST (Fig. 5), G6PD (Fig. 6), SOD (Fig. 7) and catalase (Fig. 8) between the control and *ep/ep,pe/pe* mice.
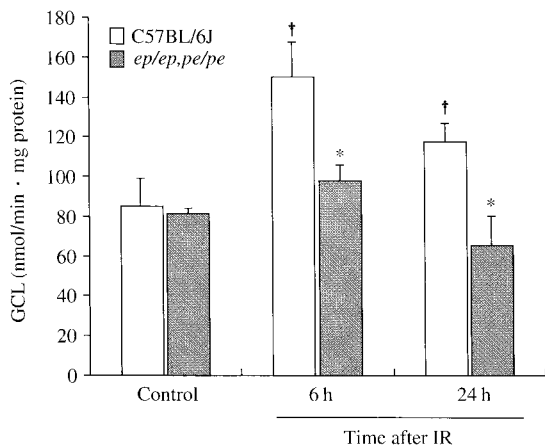
In control C57BL/6J lungs, the activities of a group
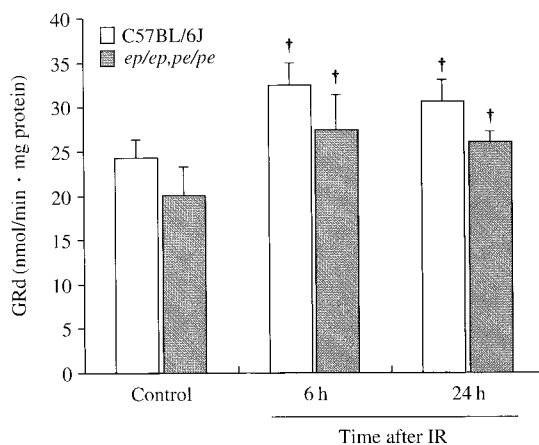
Fig. 2. Changes in GCL activity in lungs of C57BL/6J and *ep/ep,pe/pe* mice after total body irradiation. Activity of GCL was measured as described in Materials and Methods. Results are expressed as means±standard deviations (n=5). *P<0.05 vs. control C57BL/6J mice. †P<0.05 vs. respective un-irradiated control.

Fig. 4. Changes in GRd activity in lungs of C57BL/6J and *ep/ep,pe/pe* mice after total body irradiation. Activity of GRd was measured as described in Materials and Methods. Results are expressed as means±standard deviations (n=5). †P<0.05 vs. respective un-irradiated control.

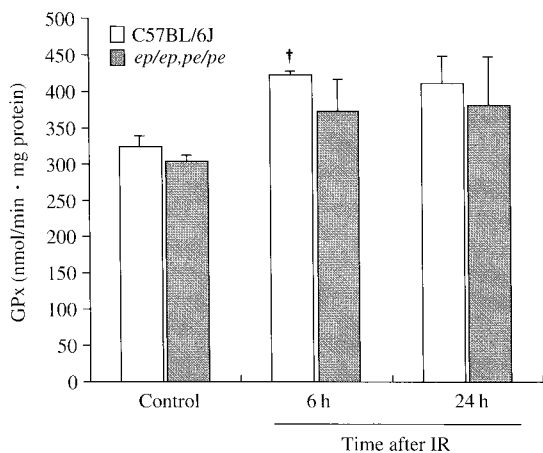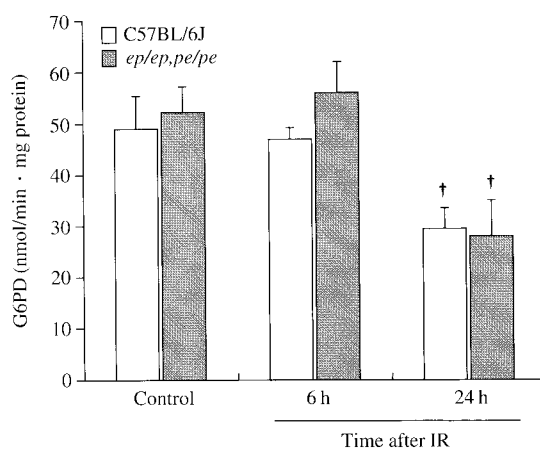**Fig. 3.** Changes in GPx activity in lungs of C57BL/6J and *ep/ep,pe/pe* mice after total body irradiation. Activity of GPx was measured as described in Materials and Methods. Results are expressed as means±standard deviations (n=5). †P<0.05 vs. respective un-irradiated control.

Fig. 5. Changes in G6PD activity in lungs of C57BL/6J and *ep/ep,pe/pe* mice after total body irradiation. Activity of G6PD was measured as described in Materials and Methods. Results are expressed as means± standard deviations (n=5). †P<0.05 vs. respective un-irradiated control.

of antioxidant enzymes, that is, GCL, GPx, GRd, and GST, all of which are involved in the glutathione metabolism in cells, were transiently increased at 6 h following irradiation. However, the transient increas-

es were not significant in *ep/ep,pe/pe* lungs except for GRd and GST. On the other hand, the activities of a second group of enzymes, that is, G6PD and catalase were decreased upon irradiation at 24 h and
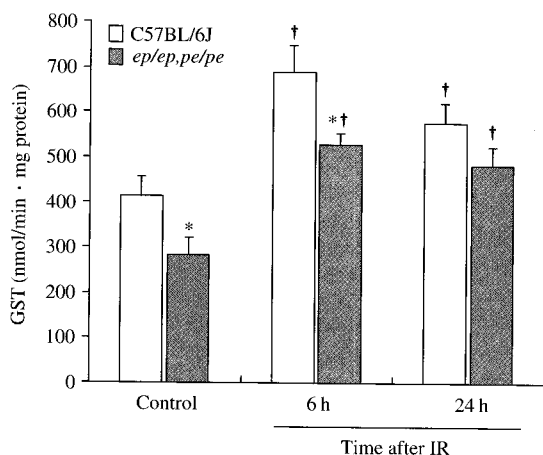
Fig. 6. Changes in GST activity in lungs of C57BL/6J and *ep/ep,pe/pe* mice after total body irradiation. Activity of GST was measured as described in Materials and Methods. Results are expressed as means ± standard deviations (n=5). *$P<0.05$ vs. control C57BL/6J mice. †$P<0.05$ vs. respective un-irradiated control.

Fig. 7. Changes in SOD activity in lungs of C57BL/6J and *ep/ep,pe/pe* mice after total body irradiation. Activity of SOD was measured as described in Materials and Methods. Results are expressed as means ± standard deviations (n=5).

6 h, respectively, in both the control and *ep/ep,pe/pe* lungs. The activities of GCL, GST, and catalase were significantly lower in *ep/ep,pe/pe* mice compared with control mice during the time course of 24 h following irradiation. The results suggest that the *ep/ep,*
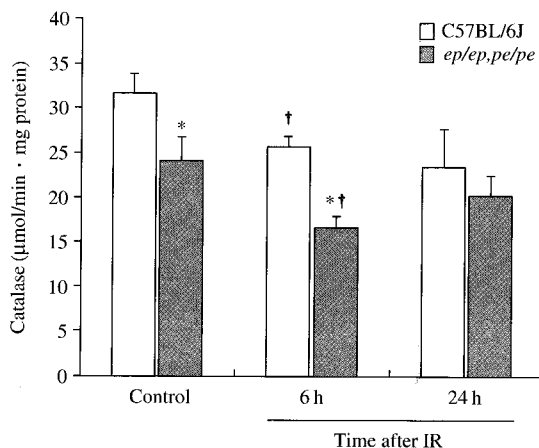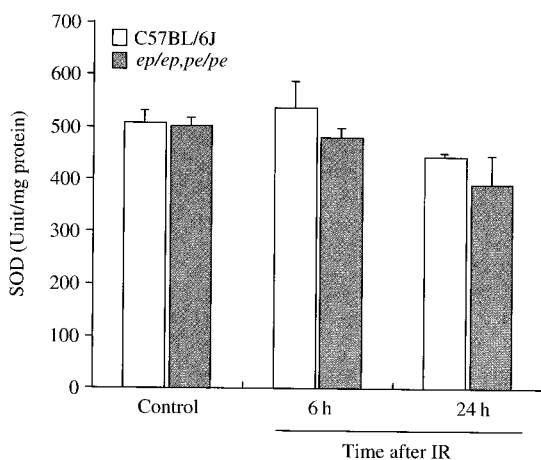

Fig. 8. Changes in catalase activity in lungs of C57BL/6J and *ep/ep,pe/pe* mice after total body irradiation. Activity of catalase was measured as described in Materials and Methods. Results are expressed as means ± standard deviations (n=5). *$P<0.05$ vs. control C57BL/6J mice. †$P<0.05$ vs. respective un-irradiated control.

pe/pe lung has incomplete adaptive response to the ionizing radiation.

DISCUSSION

The most serious clinical problem of HPS is severe lung disease that may lead to premature death. The lung abnormalities of *ep/ep,pe/pe* mutant mice resemble, in most aspects, those of human HPS patients (Lyerla *et al.*, 2003), which include significant inflammation accompanies the aberrant morphology. Inflammation is likely important in the development of lung fibrosis (Marshall *et al.*, 1997) and emphysema (Barnes, 2003). It suggests a possible role of oxidative stress and defective antioxidant system in HPS lung in early development of lung disease. In order to probe for the sensitivity of lungs of HPS patients to oxidative stress, we compared the antioxidant capacity of lungs of *ep/ep,pe/pe* mice with that of control C57BL/6J mice, and also compared the response of the antioxidant systems to the ionizing radiation. Tissue injury caused by ionizing radiation is mainly due

to the ROS generated by the interaction of ionizing radiation with water molecules. Therefore, the degree of tissue damage by irradiation, that is, radiation sensitivity, should be affected by the defense systems against ROS.

Antioxidative defenses comprise low-molecular mass agents and antioxidant proteins. Glutathione is a low molecular weight thiol present in almost all types of cells at millimolar concentration. Glutathione is synthesized by a series of enzymatic reactions, among which GCL is a key rate-limiting enzyme. Glutathione is involved in the removal of ROS by GPx in reaction with hydrogen peroxide, and also by a direct reaction with ROS without the aid of enzymes. GSH is oxidized to GSSG by the reaction with ROS. The GSSG can be removed from cells by an export in addition to a disposition by reduction by GRd. Therefore, glutathione redox cycling, that is, oxidation to GSSG by enzymatic (GPx) and nonenzymatic process and reduction back to GSH by the reaction of GRd and NADPH, which is generated by G6PD, is another important player in antioxidative defense. GSH is also consumed in the reaction with protein thiols to form mixed-disulfide under oxidative stress. Therefore, the content of cellular glutathione is determined not only by the rate of synthesis but also by the oxidation and removal under the oxidative stress. In this context, the content and oxidation ratio of cellular glutathione can serve as an indicator of the degree of oxidative stress that the cells are experiencing.

In control lungs, the free glutathione content did not decrease upon irradiation. The observation can be interpreted as a net result of balance between the removal of free cellular glutathione by oxidation and subsequent export, and also by protein-mixed disulfide formation, and increased enzymatic synthesis and salvation by recycling of glutathione under oxidative stress caused by ionizing radiation. Total body irradiation with ionizing radiation to control mice caused significant changes in antioxidant enzymes. GCL, the rate-limiting enzyme in the glutathione synthesis, and GPx, GRd, and GST, which are in-

volved in glutathione cycling, showed transient increases in the activities following irradiation. The transient increases in the above mentioned enzyme activities were also observed in the *ep/ep,pe/pe* lung with the exception of GCL. It can be considered as an adaptive response to ionizing radiation, which contributes to the maintenance of cellular glutathione pool under the oxidative stress caused by ionizing radiation. Not all the antioxidant enzymes were increased upon irradiation. G6PD, an enzyme that generates NADPH, and catalase, a peroxisomal enzyme that removes hydrogen peroxide, showed decrease in the activity upon irradiation in both control and *ep/ep,pe/pe* lungs. Deficiency in G6PD is associated with low levels of GSH and reductive metabolism in general. Therefore, low G6PD and catalase activities would tip the balance to prooxidative state. Another ROS scavenging enzyme SOD was not significantly affected by irradiation. Although antioxidant enzymes have important roles to protect cells against oxidative stress-induced damage, they themselves may also be susceptible to oxidation. Therefore, the observed changes in the enzyme activities should reflect the sum of the induction and the inactivation of the enzymes by ionizing radiation.

The *ep/ep,pe/pe* lung contains glutathione content similar to the control, however, the glutathione pool of the *ep/ep,pe/pe* lung is more oxidized than the control, indicating that the *ep/ep,pe/pe* lung is under oxidative stress even without externally exerted oxidative stress. Unlike the control lung, irradiation caused decrease in glutathione content in *ep/ep,pe/pe* lung at least for 24 h. It seems to be due to an insufficient glutathione synthesis to match the decrease in the glutathione pool under oxidative stress. The lack of GCL increase upon irradiation in *ep/ep,pe/pe* lung supports the notion. In addition, *ep/ep,pe/pe* lung showed significantly lower activity of catalase and GST compared with the control, both in steady-state and after irradiation. The decrease in the glutathione pool and antioxidant enzyme activities in *ep/ep,pe/pe* lung would significantly compromise the antioxidative capacity to cope with oxidative stress caused by

ionizing radiation.

CONCLUSION

Our results showed that *ep/ep,pe/pe* mouse lung is under consistent oxidative stress and sensitive to the oxidative stress agents due to insufficient adaptive response of antioxidant system. The poor antioxidant makeup is likely to contribute to the severe lung pathology developed in the HPS patients. Therefore, a proper antioxidant therapy might help to reduce HPS-associated lung pathogenesis and eventually to prolong the lifespan of HPS patients

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