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Susceptibility of Cigarette Smoke Condensate-Exposed Human Bronchial Epithelial Cells to Hypoxia-Reoxygenation

In-Ja Lee and Eun-Mi Choi※

Department of Chemistry, University of Incheon, Incheon 402-749, Korea

담배연기 농축액 처리에 의해 유도된 사람 기관지 상피세포주의 Hypoxia-Reoxygenation에 대한 민감성

이 인 자, 최 은 미※

인천대학교 화학과

요 약

사람 기관지상피세포주인 BEAS-2B에 담배연기농축액(CSC)을 처리하여 유도된 1198 세포주는 대조군 세포주인 1799에 비해 현저하게 낮은 glutathione 농도와 낮은 glutamate-cysteine ligase (GCL), glutathione peroxidase (GPx), glucose-6-phosphate dehydrogenase (G6PD), catalase 효소활성을 보였다. 두 세포주를 포도당 존재 하에서 4시간 hypoxia 처리 후 reoxygenation 하면서 시간에 따른 세포의 항산화계 활성을 측정한 결과, 1799 세포주에서는 의미 있는 변화가 관찰되지 않은 반면, 1198 세포주에서는 hypoxia 처리에 의해 glutathione의 농도 및 GSH/GSSG 비와 G6PD 활성이 감소되었고, reoxygenation 기에는 GPx, glutathione reductase (GRd), G6PD, superoxide dismutase 활성이 감소되었다. 그러나 reoxygenation 2시간 이후에는 GRd와 G6PD 활성의 회복이 관찰되었으며, 그 결과 GSH/GSSG 비율이 회복되었다. 이 실험 결과는 CSC가 능력을 현저히 저하시킬 수 있음을 보여준다. Glutathione은 hypoxia-reoxygenation에 의한 산화적 스트레스 하에서 항산화제로서의 역할뿐 아니라, 세포 내 GSH/GSSG 비의 변화를 통해 산화적 스트레스에 대한 항산화계의 적응 반응 여부를 결정하는 중요한 인자로 작용할 것으로 보여진다.

Key words : lung, CSC, hypoxia-reoxygenation, antioxidants, glutathione

INTRODUCTION

Cigarette smoking affects multiple organ systems resulting in numerous diseases, among which the most important are the diseases of respiratory tract such as chronic obstructive pulmonary disease and cancer. Cigarette smoke is a complex mixture of over 4,700 identified constituents, most of which are generated during the combustion process of the cigarette (Burns, 1991). Free radicals and other reactive oxygen and nitrogen species form cigarette smoke are the most important contributory factors to smoking-related diseases (Church and Pryor, 1985). Radical- and oxidant-mediated modification of cellular components such as proteins and nucleic acids and consequent damage of cells play crucial roles in the disease development. Analysis of cigarette smoke reveals that tar contains $>10^{17}$ long-lived radicals per gram, and the

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

volatile fraction contains $>10^{15}$ short-lived reactive radicals per gram (Pryor and Stone, 1993). Aerobic life forms have developed a range of defense mechanisms that counteract alterations of the physiological state occurring in the course of damage. Cigarette smoke chemicals interfere with these mechanisms in a two-fold fashion. First, smoke chemicals increase the number of repairs required from the defense machinery by increasing the number of damaged sites. Second, smoke chemicals interfere with the defense machinery itself and reduce its efficiency. Consequently, cigarette smoking exhausts cellular defense and repair functions. The lung is the only organ that has the highest exposure to atmospheric oxygen. In order to cope with the oxidative stress caused by reactive oxygen species (ROS) generated either endogenously by metabolic reactions, such as from mitochondrial electron transport during respiration, and exogenously from agents such as air pollutants and chemicals, the lung is endowed with a battery of endogenous antioxidants, both enzymatic and non-enzymatic.

In this study, we evaluated the effect of hypoxia-reoxygenation on the antioxidant system in cigarette smoke condensate (CSC)-exposed human bronchial epithelial cells. CSC or tar is the particulate component of the cigarette smoke, which contains numerous long-lived free radical species and has been shown to be involved in the lung carcinogenesis (Rodgman *et al.*, 2000). Epithelial cells in respiratory tract are normally well oxygenated, but may be exposed to hypoxia due to the airway obstruction in many pathological conditions such as pulmonary edema, acute respiratory distress syndrome, chronic obstructive pulmonary diseases, and obstructive sleep apnea. Chronic intermittent hypoxia, that is, repeated hypoxia and reoxygenation, plays a significant role in pathogenesis and progression of these diseases. The rapid burst of ROS generation by reoxygenation following hypoxia is well documented (Courtois *et al.*, 1998). However, hypoxic cells also increase paradoxically the production of ROS, leading to oxidative stress. The primary source for ROS generation in cells during hypoxia has been reported to be complex III in mitochondrial transport chain (Guzy and Schumacker, 2006). In addition, ROS release might be enhanced during hypoxic conditions by the activation of xanthine oxidase (Sohn *et al.*, 2003), NADPH oxidase (Jones *et al.*, 2000), and phopholipase A2 (Neidlinger *et al.*, 2005).

The antioxidant system we analyzed includes glutathione that is the major small molecular thiol antioxidant; glutamate cysteine ligase (GCL) that is a rate-limiting enzyme for glutathione synthesis; glutathione peroxidase (GPx), glutathione reductase (GRd), and glucose-6-phosphate dehydrogenase (G6PD) that are involved in glutathione recycling metabolism; and superoxide dismutase (SOD) and catalase that are ROS scavenging enzymes. Our results show that adversely compromised glutathione metabolism in CSC-exposed bronchial cells is the major factor responsible for their susceptibility to oxidative stress excerted by hypoxia-reoxygenation.

MATERIALS AND METHODS

1. Materials

Glutathione (reduced, GSH; oxidized, GSSG), 2,4-dinitrofluorobenzene (FDNB), NADPH, $NADP^+$, NADH, ATP, phosphoenolpyruvate, glucose-6-phosphate, xanthine, GRd, pyruvate kinase (PK), lactate dehydrogenase (LDH), xanthine oxidase (XO), and cytochrome c were purchased from Sigma (St. Louis, MO, USA). Supelcosil^{TM} LC-NH_2 column (particle size 5 μm, 25 cm × 4.6 mm) was from SUPELCO (Bellefonte, PA, USA).

2. Cells and cell culture

As a model for the study of the effect of cigarette smoking on the hypoxia-reoxygenation-induced oxidative stress to human lung, we used cell lines derived from BEAS-2B cell line, which is a human bronchial epithelial cell immortalized with a hybrid Adenovirus/ Simian Virus 40. Klein-Szanto *et al.* used a xenotransplantation system in which the BEAS-2B cells were grown in deepithelialized rat tracheas that

were transplanted subcutaneously into athymic nude mice. 1799 is a non-tumorigenic cell line that was derived from BEAS-2B cells exposed to a beeswax pellet alone, and 1198 is non-tumorigenic cell line that was derived from the cells exposed to beeswax pellet containing CSC (Klein-Szanto *et al.*, 1992). The cell lines were obtained from Dr. Y. M. Park (Roswell Park Cancer Institute, Buffalo, NY, USA). The cells were cultured in RPMI 1640 (GIBCO-BRL, Richmond, USA) supplemented with 10% fetal calf serum at 37˚C in an atmosphere of 5% CO_2-95% air. The glucose concentration was 2 g/L for all experiments including hypoxia treatment.

3. Hypoxia-reoxygenation treatment

Cells were subjected to hypoxia by oxygen deprivation in a hypoxic chamber (Forma Scientific, Marietta, USA) as previously described (Baek *et al.*, 2001). The medium was pre-equilibrated before each experiment with hypoxic gas mixture containing 5% CO_2, 85% N_2 and 10% H_2 at 37˚C. Cells were deprived of oxygen by triple changes of the culture medium with deoxygenated medium. The oxygen concentration in the hypoxic chamber and in the exposure medium was monitored by an oxygen indicator (Forma Scientific, Marietta, USA). The oxygen concentration was maintained at lower than 0.05%. The normoxic control cells were maintained in a 37˚C incubator with 5% CO_2-95% air. After exposure to hypoxia for 4 h, cell cultures were reoxygenated under normoxic condition for the indicated times. Cells of hypoxia-only group were harvested in the hypoxic chamber at the end of 4 h of hypoxia treatment and stored with nitrogen gas in tubes. Cell samples were stored at −80˚C until analyzed.

4. Glutathione analysis

Glutathione was measured by using HPLC as described previously (Park *et al.*, 1998). Briefly, 5% perchloric acid extract of the cells was derivatized with FDNB, and GSH and GSSG were separated by HPLC on a Supelcosil^{TM} LC-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.

5. Enzyme activity analyses

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).

6. Statistical analysis

Data are expressed as means±standard deviations (SD) from five samples in each group. The significance of the differences between experimental and

Table 1. Basal levels of antioxidants in 1799 and 1198 cells

	1799	1198
Glutathione (nmol/mg protein)	49.8±5.7	31.6±2.6**
GSH/GSSG	24.4±1.6	22.3±2.8
GCL (nmol/min · mg protein)	147.2±11.9	89.7±7.4***
GPx (nmol/min · mg protein)	68.6±7.2	55.6±1.0**
GRd (nmol/min · mg protein)	14.4±1.1	13.4±0.9
G6PD (nmol/min · mg protein)	195.5±12.7	133.7±11.7***
SOD (unit/mg protein)	121.2±1.5	134.1±13.2
Catalase (μmol/min · mg protein)	4.92±0.44	4.04±0.38**

*$P<0.05$; **$P<0.01$; ***$P<0.001$

control groups was determined using the unpaired Student's t-test. P values of <0.05 were considered as significant.

RESULTS

1. Basal levels of antioxidants in 1799 and 1198 cells

We compared basal levels of antioxidants in 1198 cells, the cell line derived from BEAS-2B cells exposed to beeswax pellet containing CSC, with those in 1799 cells, the cell line derived from the cells exposed to a beeswax pellet alone (Table 1). 1198 cells showed significantly lower glutathione content than 1799 cells. On the other hand, similar GSH/GSSG ratio was observed in the two. The 1198 showed significantly lower activity of GCL, the rate limiting enzyme for glutathione synthesis, suggesting that lower glutathione content observed in 1198 was due to slowdown of glutathione synthesis. In addition, among the enzymes that participate in the glutathione recycling, GPx and G6PD were significantly lower in 1198. Catalase activity was also low in 1198 cells. The results clearly show that CSC-exposed 1198 cells are low in antioxidant capacity. The basal oxidative stress level in 1198, however, seems to be similar to that in 1799 at normal growth condition, because difference in the ratio of GSH/GSSG was not observed between the two.

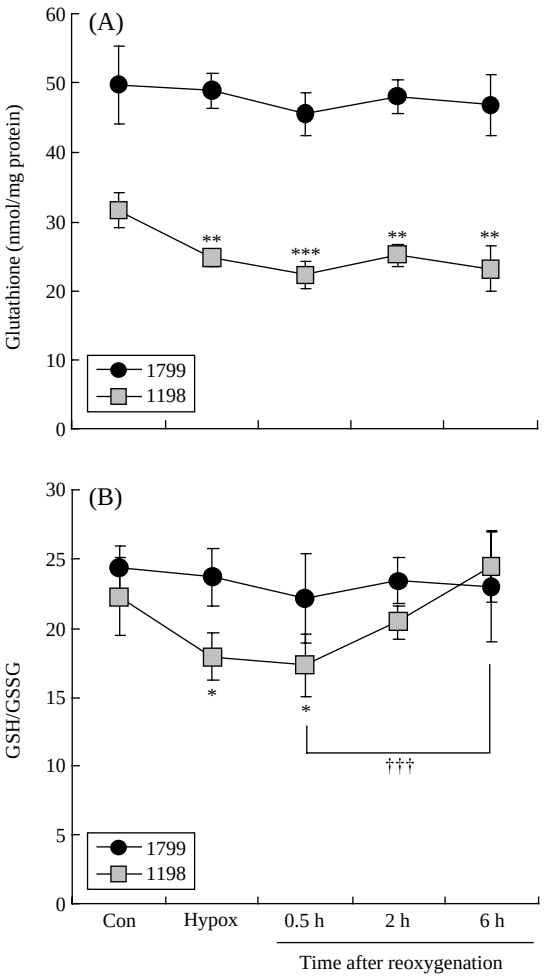

Fig. 1. Changes in glutathione, (A) total glutathione and (B) GSH/GSSG ratio, in 1799 and 1198 cells after hypoxia and reoxygenation. The acid extracts of the cells were derivatized with FDNB, and glutathione content was analyzed by the HPLC method. Total glutathione was expressed as the GSH equivalent to the sum of GSH and GSSG. Results are expressed as means±SD (n=5). *$P<0.05$, **$P<0.01$, ***$P<0.001$, vs. respective normoxic control. $^{\dagger\dagger\dagger}P<0.001$ vs. 0.5 h of reoxygenation.

2. Changes in glutathione by hypoxia-reoxygenation

Having found the lower antioxidant capacity in 1198, we then examined the effect of hypoxia and reoxygenation on 1198 and 1799 cells. Glutathione is

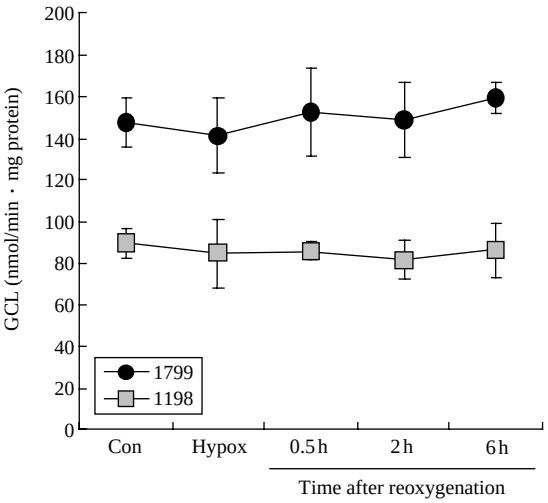

Fig. 2. Changes in GCL activity in 1799 and 1198 cells after hypoxia and reoxygenation. Activity of GCL was measured as described in Materials and Methods. Results are expressed as means ± SD (n=5).

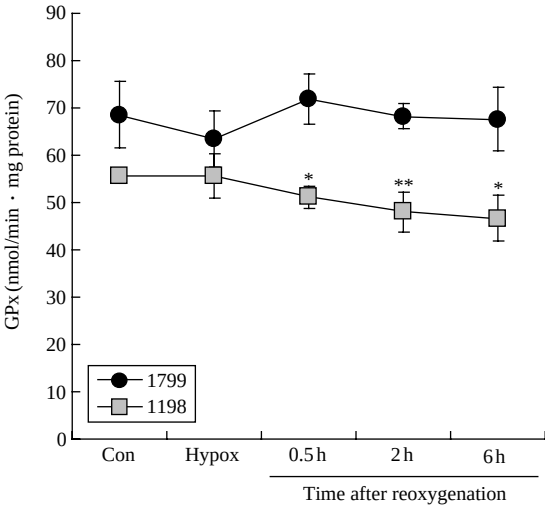

Fig. 3. Changes in GPx activity in 1799 and 1198 cells after hypoxia and reoxygenation. Activity of GPx was measured as described in Materials and Methods. Results are expressed as means ± SD (n=5). $*P<0.05$, $**P<0.01$, vs. respective normoxic control.

not only the important antioxidant molecule, but it is also recognized as a sensitive indicator of oxidative stress. Under oxidative stress, glutathione (GSH) is oxidized to disulfide form (GSSG) by enzymatic and nonenzymatic reactions with ROS, and the GSSG is subsequently excreted from cells. Therefore, cellular glutathione level reflects a steady state balance between synthesis and loss. In 1799 cells, neither the total glutathione content nor the GSH/GSSG ratio was significantly affected by hypoxia and reoxygenation (Fig. 1). On the other hand, in 1198 cells, 4 h of hypoxia treatment resulted in significantly lower glutathione content and GSH/GSSG ratio. In the reoxygenation phase, the 1198 cells were continuously depleted of glutathione up to 6 h following reoxygenation. However, the GSH/GSSG ratio in 1198 cells started to bounce back after 2 h and was completely restored after 6 h of reoxygenation.

3. Changes in the enzymes for glutathione synthesis and recycling by hypoxia-reoxygenation

To explain the behavior of glutathione pool upon hypoxia-reoxygenation in 1799 and 1198 cells, we measured activities of enzymes for glutathione meta-

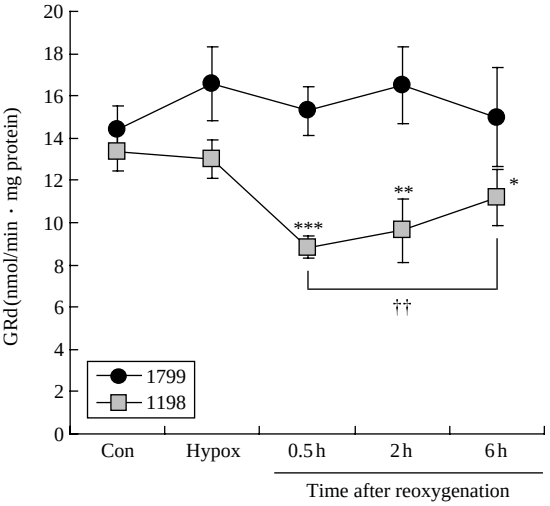

Fig. 4. Changes in GRd activity in 1799 and 1198 cells after hypoxia and reoxygenation. Activity of GPx was measured as described in Materials and Methods. Results are expressed as means ± SD (n=5). $*P<0.05$, $**P<0.01$, $***P<0.001$, vs. respective normoxic control. $^{\dagger\dagger}P<0.01$ vs. 0.5 h of reoxygenation.

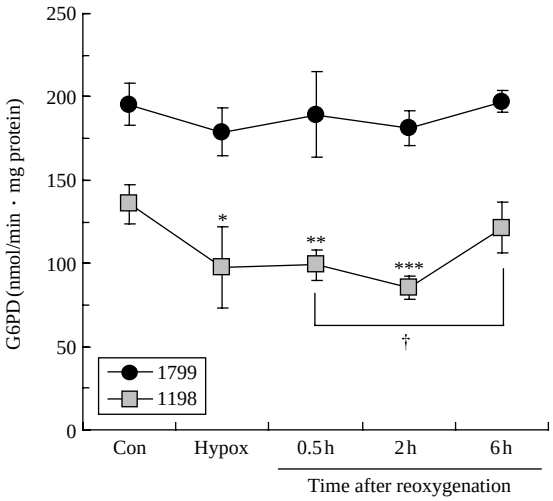

Fig. 5. Changes in G6PD activity in 1799 and 1198 cells after hypoxia and reoxygenation. Activity of G6PD was measured as described in Materials and Methods. Results are expressed as means ± SD (n=5). $*P<0.05$, $**P<0.01$, $***P<0.001$, vs. respective normoxic control. $^{\dagger}P<0.05$ vs. 0.5 h of reoxygenation.

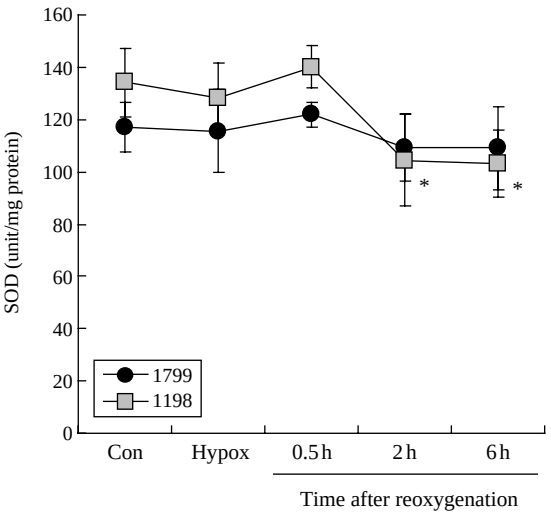

Fig. 6. Changes in SOD activity in 1799 and 1198 cells after hypoxia and reoxygenation. Activity of SOD was measured as described in Materials and Methods. Results are expressed as means ± SD (n=5). $*P<0.05$ vs. respective normoxic control.

bolism. GCL did not show significant change in activity in either 1799 or 1198 cells throughout the treatment (Fig. 2). However, a clear difference in the activities of enzymes involved in glutathione recycling, that is, GPx, GRd and G6PD, between 1799 and 1198 cells was observed (Figs. 3-5). In 1799 cells, none of the enzyme activities was significantly changed throughout the treatment. The results suggest that the glutathione metabolism of 1799 cells is sufficiently efficient even under the presence of increased ROS by hypoxia-reoxygenation to maintain the cellular glutathione pool. On the other hand, in 1198 cells, all three enzyme activities were significantly affected. GPx activity was significantly decreased in the phase of reoxygenation, and the decrease persisted up to 6 h (Fig. 3). GRd activity was also decreased in reoxygenation phase, but the activity started to recover after 2 h (Fig. 4). While the activities of GPx and GRd were decreased in the phase of reoxygenation, G6PD showed significant decrease in the phase of hypoxia, and the activity recovered after 2 h following reoxygenation (Fig. 5).

4. Changes in SOD and catalase by hypoxia-reoxygenation

The activities of SOD (Fig. 6) and catalase (Fig. 7) in 1799 cells did not show statistically significant changes throughout the hypoxia-reoxygenation treatment. In 1198 cells, SOD activity was decreased after 2 h and did not recover up to 6 h following reoxygenation, although the catalase activity was not changed.

DISCUSSION

In this study, we evaluated the response of antioxidant system in CSC-exposed human bronchial epithelial cells (1198 cells) to the hypoxia and reoxygenation. Hypoxia-reoxygenation has been implicated in the pathogenesis of many lung diseases. Given the compelling evidence that hypoxia and reoxygenation generate ROS, the relative ratio between the degrees of oxidative damage to the cells and the degrees of protection of the cells from the oxidative damage in respiratory tract will determine the fate of the cells

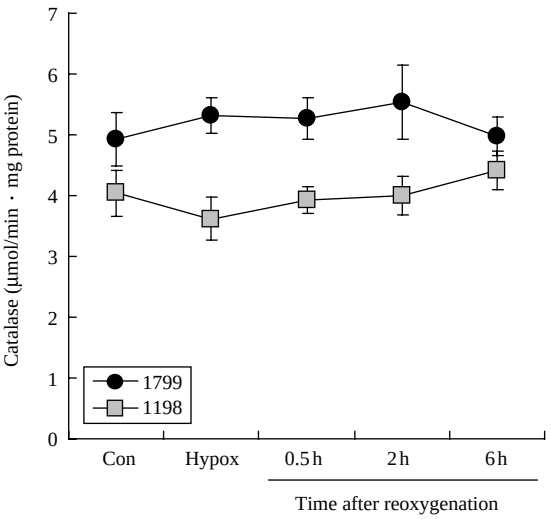

Fig. 7. Changes in catalase activity in 1799 and 1198 cells after hypoxia and reoxygenation. Activity of catalase was measured as described in Materials and Methods. Results are expressed as means ± SD (n=5).

under such pathological conditions.

1198 cells, which were derived form BEAS-2B cells exposed to CSC, displayed adversely compromised antioxidant system. This observation strongly supports the notion that cigarette smoking causes depletion of antioxidant capacity. In particular, 1198 cells showed decreased steady state levels of glutathione content and activities of GCL, GPx, G6PD and catalase compared with control 1799 cells. It is presumed to be resulted from accumulation of CSC-originated damages on redox-sensitive molecular targets including proteins in signaling pathways, eventually leading to the alteration of expression of antioxidant proteins. Down-regulation of many genes that are mainly associated with xenobiotic metabolism, DNA damage and repair, and inflammatory responses has been identified in smokers' peripheral lymphocytes (Lodovici *et al.*, 2007). Despite the lower antioxidnats, 1198 cells maintained a steady state level of GSH/GSSG ratio similar to that in 1799, indicating that under normal condition, their antioxidant system is not overloaded with oxidants. Many reports suggest that the GSH/GSSG ratio might provide a sensitive indicator of oxidative stress (Park *et al.*, 1998; Avanzo *et al.*, 2001). Considering the importance of glutathione for antioxidant function, however, it is expected that the 1198 cells may suffer from oxidative damage, when ROS increase further.

In our experiment of the hypoxia-reoxygenation treatment, that is, 4 h hypoxia in the presence of glucose in media and subsequent reoxygenation under normoxic condition, the 1799 cells did not seem to suffer from increased oxidative damage, which was evidenced by the maintenance of glutathione content and GSH/GSSG ratio. The antioxidant enzyme activities, including the enzymes in the glutathione metabolism and ROS-scavenging SOD and catalase, also were not changed significantly. The results suggest that under the conditions of hypoxia-reoxygenation in our experiment, the balance between oxidants and antioxidants is still holding in 1799 cells. On the contrary, 1198 cells showed sustained depletion of glutathione and significantly decreased activities of GPx, GRd, G6PD, and SOD after hypoxia-reoxygenation. The measured activity of enzyme under oxidative stress is a net value from the sum of the loss of activity due to catalytic inhibition and the increase of activity due to adaptive response, because the antioxidant enzyme themselves are redox sensitive. Inhibition of several antioxidant enzymes containing redox-sensitive active sites due to the oxidative damage has been reported (Tabatabaie and Floyd, 1994; Riganti *et al.*, 2003; Hu *et al.*, 2007). Our results suggest that in 1198 cells, oxidative damage prevails under hypoxia-reoxygenation, causing inhibition of the enzymes and, as a result, depletion of glutathione.

Although the persistence of oxidatively stressed state during the hypoxia-reoxygenation was evident in 1198 cells, in which a sustained decrease of glutathione content was observed, their antioxidant system started to recover at the time of 2 h after reoxygenation, at which the activity of GRd and G6PD started to bounce back. The increase in the GRd and G6PD activities, presumably as an adaptive response, seems to be associated with the decrease in GSH/GSSG ratio. Requirement of particular ratio of GSH/GSSG has

been reported in NF-κB, AP-1, and several other transcription factors (Bandyopadhyay *et al.*, 1998; Rokutan *et al.*, 1998; Klatt *et al.*, 1999). The recovery of GRd and G6PD activities then leaded to the recovery of GSH/GSSG ratio. From our results, it can be speculated that in the lung cells with adversely compromised antioxidant system due to the cigarette smoking, hypoxia-reoxygenation, as in many pathological conditions, will initially cause loss of antioxidant enzyme activities and oxidation and depletion of glutathione. And in later phase, depending on the intensity and duration of the oxidative stress, adaptive response that is possibly facilitated by the lowered GSH/GSSG ratio may restore the antioxidant capacity. Therefore, in the case of cigarette smoking-related diseases, glutathione not only functions as a key antioxidant but also seems to play an important role in the adaptive response of antioxidant system including its own metabolism.

CONCLUSION

Our results show that 1198 cells, which were derived from CSC-exposed BEAS-2B cells, have significantly lower antioxidant capacity compared with 1799 cells, the control cell line. The 1198 cells are more susceptible to oxidative stress caused by hypoxia-reoxygenation, showing loss of antioxidant enzyme activities and oxidation and depletion of glutathione. In the later phase of reoxygenation, however, the antioxidant system in 1198 cells, that is, GRd and G6PD, started to recover, and as a result, restoration of GSH/GSSG ratio was accomplished. Our results also suggest that glutathione, especially GSH/GSSG ratio, plays an important role as a sensitive indicator for oxidative stress and might be involved in adaptive response of the antioxidant system including its own metabolism.

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