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Original Contribution

OXIDATION OF GLUTATHIONE AND CYSTEINE IN HUMAN PLASMA ASSOCIATED WITH SMOKING

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Abstract—Cigarette smoking contributes to the development or progression of numerous chronic and age-related disease processes, but detailed mechanisms remain elusive. In the present study, we examined the redox states of the GSH/GSSG and Cys/CySS couples in plasma of smokers and nonsmokers between the ages of 44 and 85 years (n = 78 nonsmokers, n = 43 smokers). The Cys/CySS redox in smokers (-64 ± 16 mV) was more oxidized than nonsmokers (-76 ± 11 mV; $p < .001$), with decreased Cys in smokers (9 ± 5 μM) compared to nonsmokers (13 ± 6 μM; $p < .001$). The GSH/GSSG redox was also more oxidized in smokers (-128 ± 18 mV) than in nonsmokers (-137 ± 17 mV; $p = .01$) and GSH was lower in smokers (1.8 ± 1.3 μM) than in nonsmokers (2.4 ± 1.0; $p < .005$). Although the oxidation of GSH/GSSG can be explained by the role of GSH in detoxification of reactive species in smoke, the more extensive oxidation of the Cys pool shows that smoking has additional effects on sulfur amino acid metabolism. Cys availability and Cys/CySS redox are known to affect cell proliferation, immune function, and expression of death receptor systems for apoptosis, suggesting that oxidation of Cys/CySS redox or other perturbations of cysteine metabolism may have a key role in chronic diseases associated with cigarette smoking. © 2003 Elsevier Inc.

Keywords—Oxidative stress, Oxidation-reduction potential, Aging, Free radicals

INTRODUCTION

Considerable evidence indicates that oxidative stress contributes to age-related diseases affecting major organ systems, such as atherosclerosis, chronic lung disease, age-related macular degeneration, and Alzheimer's disease [1–4]. Cigarette smoking has been associated with increased risk of these diseases and cigarette smoke induces oxidative stress. However, because the diseases are complex and affected by multiple genetic, environmental, and dietary factors, the precise mechanisms involved in promotion of these chronic diseases by cigarette smoking remain unclear [5–7].

Oxidative stress is associated with low antioxidant levels, lipid peroxidation, protein modification, and DNA damage [8–11]. Among these, a decline in the endogenous glutathione-dependent detoxification systems has been most consistently observed in in vitro studies, animal models, and clinical studies [12]. Glutathione (GSH) is a major antioxidant used to eliminate peroxides and to maintain vitamins C and E in their reduced and functional forms [13]. The steady-state balance of GSH and its oxidized disulfide form (GSSG) are expressed as the redox state (E_h) of the GSH/GSSG couple, calculated using the Nernst equation. This value provides a dynamic and quantitative measure of oxidative stress [14]. The redox state of the GSH/GSSG couple is oxidized in human plasma in association with aging [15], chemotherapy [16], and diabetes [17], suggesting that GSH/GSSG redox may provide a useful link between in vitro mechanistic studies of oxidative stress and in vivo studies of smoking and disease.

GSH is synthesized from cysteine (Cys), glycine, and glutamate, with Cys availability often being a limiting factor for the rate of synthesis [18]. The predominant form of Cys in the plasma is the oxidized disulfide form, cystine (CySS), which is typically present at concentrations greater than 40 μM, compared to 8–10 μM for Cys

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[19]. Many cell types lack a transport system for CySS and, therefore, are dependent solely upon uptake of Cys to support cellular needs for Cys [20,21]. Because of the relatively high CySS concentration, the Cys/CySS redox state in human plasma (about −80 mV) is considerably oxidized compared to GSH/GSSG redox (about −140 mV) [18]. Although the basis for this difference remains unclear, recent studies show that cell proliferation rate is dependent upon the redox state of Cys/CySS in the culture medium [22,23] and that a more reduced redox state is associated with resistance to apoptosis [24].

Because cigarette smoke contains compounds (e.g., acrolein) that react with GSH [25], as well as reactive oxygen species (ROS) that oxidize GSH to GSSG, we have determined whether the plasma concentrations of GSH and Cys are decreased and the GSH/GSSG and Cys/CySS oxidized in smokers compared to nonsmokers. The study was designed to study individuals older than 45 years of age because this population is at greatest risk of oxidative stress [15] and chronic disease [26]. The results confirm the aforementioned hypotheses regarding GSH and Cys concentrations and GSH/GSSG and Cys/CySS redox couples in smokers compared to nonsmokers. Because chemical detoxification, cell proliferation, and apoptosis are dependent upon these biochemical parameters, these changes may provide both a means to quantify risk of disease and a mechanistic basis for enhanced systemic toxicities associated with smoking.

MATERIALS AND METHODS

Materials

Sodium heparin, bathophenanthroline disulfonate sodium salt (BPDS), iodoacetic acid, dansyl chloride, L-serine, γ-glutamylglutamate (γ-Glu-Glu), GSH, GSSG, Cys, CySS, and sodium acetate trihydrate were from Sigma Chemical Corp. (St. Louis, MO, USA). The mixed disulfide of Cys and GSH, CySSG, was from Toronto Research Chemicals (Toronto, Ont., Canada). Boric acid, sodium tetraborate, potassium tetraborate, perchloric acid, and acetic acid were reagent grade and purchased locally. Methanol, acetone, and chloroform were HPLC grade.

Study population and recruitment

Measurements of plasma glutathione (GSH) and glutathione disulfide (GSSG), as well as cysteine (Cys) and cystine (CySS), were obtained from current smokers and nonsmokers between the ages of 44 and 85 years ($n = 78$ nonsmokers, $n = 43$ smokers). Samples were collected between December of 2000 and May of 2002. Diabetics and subjects with age-related macular degeneration were excluded from the study because previous research has shown that GSH/GSSG redox is oxidized in association with type 2 diabetes and age-related macular degeneration [17]. There were no other disease exclusion criteria. Blood samples were collected from the Emory Eye Center, the Emory General Clinical Research Center, and the Atlanta Veterans Affairs Hospital. Subjects included patients, patient families, study site workers, and any other volunteers who had seen the advertisements. Volunteers were asked to fill out a one-page questionnaire regarding their age, sex, race, smoking status (never, previous, current), physical activity, diet, health status, and alcohol consumption. The study and recruitment procedures were approved by the Emory Investigational Review Board, and informed consent was obtained from all participants.

Sample collection. Blood was collected with syringes and 23-gauge heparinized butterfly needles; blood (0.5 ml) was immediately transferred to microcentrifuge tubes containing 0.5 ml of a redox preservation solution [27]. Tubes were centrifuged to remove blood cells, and 200 μl aliquots were transferred to 200 μl of 10% perchloric acid with 0.2 M boric acid and 10 μM γ-Glu-Glu (internal standard). This procedure enables quantitative recovery of Cys, CySS, GSH, and GSSG, and minimizes variation in redox state between sampling and acidification. Samples were stored at −80°C before derivatization (less than 2 months) and analyzed by HPLC with fluorescence detection [27].

Redox state calculations

The redox potentials (E_h) of the different thiol/disulfide couples were calculated using the Nernst equation, $E_h = E_o + RT/nF(\ln[\text{disulfide}]/[\text{thiol}]^2)$, where E_o is the respective standard potential for the redox couple at pH 7.4, R is the gas constant, T is the absolute temperature, n is 2 for the number of electrons transferred, and F is Faraday's constant. The respective standard potentials for the Cys/CySS and GSH/GSSG couples at pH 7.4 were −250 mV and −264 mV [27].

Statistical methods

The distributions of metabolites and redox parameters were described using boxplots, histograms, and summary statistics, and were compared to a normal distribution using the Anderson-Darling test. Factors not normally distributed were transformed for statistical analyses (natural log, square root, square). A particular transformation was chosen for a factor based on producing a nonsignificant normality test with the largest Anderson-Darling test statistic. The specific transformation used for each of the metabolites and redox parameters were: GSH, GSH_t (total GSH plus disulfide forms), Cys, CySS, and

CySSG, square root; CySSG, natural log; E_h for Cys/CySS, square. E_h for GSH/GSSG did not require transformation. Mean values of the transformed values of the metabolites for smokers and nonsmokers were compared using an independent groups *t*-test. In all cases, the *p* values reported are for the statistical tests using the transformed data. However, the graphs of the distributions and the summary statistics of the metabolites and redox parameters are given in terms of the nontransformed values. In the text, the metabolites and redox parameters are presented as mean ± standard deviation. Subject characteristics were compared between smokers and nonsmokers using an independent groups *t*-test or a chi-square test depending on the metric of the characteristic. The effect of smoking, adjusted for other subject characteristics, was evaluated using a two-factor ANOVA model. Separate models were fit for each metabolite as the dependent variable with smoking and each of the patient characteristics as the independent variables. A linear regression model (without interaction terms) was used to evaluate the effect of smoking while simultaneously controlling for the effect of all other patient characteristics. A p value $<.05$ was considered statistically significant in all analyses.

RESULTS

Subject characteristics

The study included 121 nondiabetic adults between the ages of 44 and 85 years consisting of 43 smokers and 78 nonsmokers. The mean number of years smoked in the smokers was 33 ± 12 years and the range was 4–55 years. Of this group, 86% smoked for more than 20 years. The nonsmokers consisted of those who had never smoked ($n = 20$) and those who had previously smoked ($n = 58$). Previous smokers had stopped smoking more than 2 years previously. Initial analyses showed no significant differences between those who never smoked and those who had previously smoked (see below). Thus, those who never smoked and those who previously smoked were combined to form a nonsmoking group that was used for all statistical analyses.

Comparisons of the smoking and nonsmoking groups showed that they did not differ in alcohol use, exercise type, or exercise frequency, but differed in mean age, sex, race, and supplement use (Table 1). The mean age of smokers (58 years) was significantly lower than that of nonsmokers (63 years; $p < .005$). However, oxidation of GSH/GSSG and Cys/CySS increases with age [15] and smokers were younger than nonsmokers; therefore, the difference in mean age could not explain a more oxidized redox state in smokers.

Table 1. Characteristics of Subjects According to Smoking Status, Age, Gender, Race and Supplement Use

Subject Characteristics	Current Smoking Status		*p* value
	No ($n = 78$)[a]	Yes ($n = 43$)	
Age (mean ± SD)	63 ± 9	58 ± 6	.003
Gender (% male)	40	58	.05
[b]Race (% white)	79	56	.008
[c]Supplements (% supplemented)	44	14	.01

[a] Subjects characterized as nonsmokers included 20 subjects who reported having never smoked and 58 subjects who did not currently smoke and had not smoked for at least 2 years.

[b] There was a total of 33 blacks, 1 Asian, and 1 other race included in the study population.

[c] Taking vitamin C, E, beta carotene, any other antioxidant or a multivitamin supplement were considered supplements.

In the current study there was a significant difference in gender between the two groups (58% of smokers were male; 40% of nonsmokers were male) that warrants specific examination of possible effect of gender. There was also a significant difference in race between the smoking group (56% Caucasian) and nonsmoking group (79% Caucasian), but the numbers of individuals are relatively small for evaluation of possible effects of race. There was a large and potentially important difference in supplement use, with only 14% of smokers reporting dietary supplement use compared to 44% for nonsmokers. The supplements consumed by the subjects included multivitamins and antioxidants such as vitamin C, vitamin E, and beta carotene. None of the supplements used by the subjects contained any Cys pro-drugs such as N-acetylcysteine (NAC). Thus, because of these differences between smokers and nonsmokers, analyses were initially conducted to compare the total smoking and nonsmoking populations and subsequently analyzed with adjustments for age, sex, race, and supplement use.

Effect of smoking on metabolite concentrations

The plasma concentration of GSH in the nonsmoking group was 2.4 ± 1.0 μM, a value similar to previous studies [18]. The mean value in smokers was significantly lower, 1.8 ± 1.3 μM ($p < .005$; Fig. 1A). There was no significant difference in GSSG concentration (smokers: 0.2 ± 0.3 μM; nonsmokers 0.2 ± 0.2 μM; Fig. 1B), but the mixed disulfide of GSH and Cys, CySSG, was significantly lower in smokers (Fig. 1C). The total GSH (GSH_t) calculated as GSH + CySSG + 2 × GSSG, was also significantly lower in smokers than in nonsmokers (Fig. 1D). When previous smokers were compared to those who had never smoked, there was no significant difference between the two groups (data not shown).

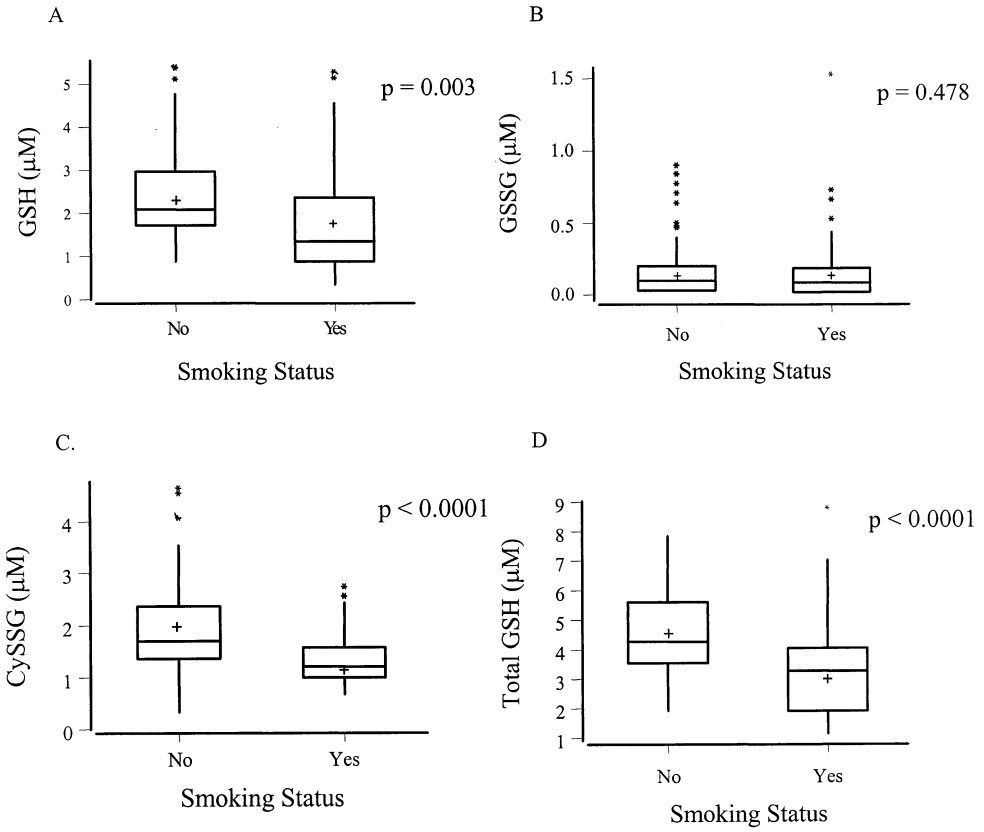

Fig. 1. Data are given as box plots with mean represented by + and median represented by the line within the box. The ends of the box represent the 25th and 75th quartiles. The vertical line represents all data within 3 interquartiles from the box. The * marks values outside 3 interquartiles. Comparison of GSH and related parameters (GSSG, CySSG, and GSH_t in current smokers and nonsmokers (previous smokers and nonsmokers). (A) Glutathione was decreased in smokers (1.8 ± 1.3 μM; $n = 43$) compared to nonsmokers (2.4 ± 1.0 μM; $n = 77$). (B) There was no significant difference in GSSG (smokers: 0.17 ± 0.27 μM; nonsmokers: 0.17 ± 0.20 μM). (C) Total GSH (GSH_t) was lower in smokers (3.3 ± 1.7 μM) compared to nonsmokers (4.5 ± 1.4 μM). (D) The disulfide of GSH and Cys, CySSG, was lower in smokers (1.3 ± 0.5 μM) than nonsmokers (1.9 ± 0.9 μM).

Results similarly showed that mean plasma Cys concentration was lower in smokers (9 ± 5 μM) compared to nonsmokers (13 ± 6 μM; $p < .0001$; Fig. 2A). CySS was also present at a lower concentration in smokers (86 ± 30 μM) than nonsmokers (100 ± 29 μM; $p < .01$; Fig. 2B). Therefore total cysteine, (Cys_t) was lower in smokers (182 ± 62 μM) than in nonsmokers (215 ± 57 μM; $p < .005$; Fig. 2C). When previous smokers were examined in relation to those who never smoked, there was no significant difference between the two groups (data not shown).

Thiol-disulfide redox states in cigarette smokers

To determine whether the thiol/disulfide redox states of GSH/GSSG and Cys/CySS were more oxidized in smokers, E_h values were calculated using the Nernst equation. The GSH E_h value for nonsmokers was −137 ± 17 mV, a value similar to that previously observed for healthy individuals [18]. In contrast, the GSH/GSSG couple was more oxidized in the smokers (−128 ± 18 mV; $p = .01$; Fig. 3A). A large difference was also seen between nonsmokers and smokers for E_h Cys/CySS. The value for nonsmokers was −76 ± 11 mV, whereas the value for smokers was −64 ± 16 mV ($p < .001$; Fig. 3B). These results show that in addition to smokers having a smaller total pool size for these thiol-disulfide couples, the redox states for these metabolic pools were more oxidized.

Analysis of subject characteristics

Given that there were significant differences between the smokers and nonsmokers in age, sex, race, and supplement use (Table 1), analyses were performed to determine whether these differences could account for the significant associations of smoking with metabolite concentrations and redox state (Table 2).

There was no significant interaction between age and smoking status for any of the metabolites except the mixed disulfide (CySSG). For CySSG, the nonsmokers demonstrated an increase in mean concentration with

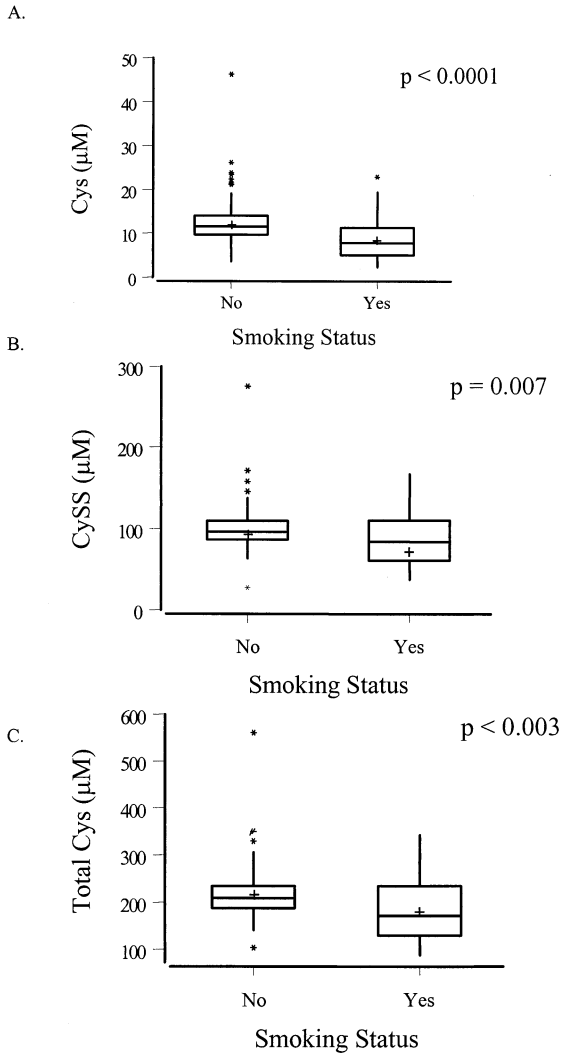

Fig. 2. Comparison of cysteine (Cys) and cystine (CySS) concentration in plasma of current smokers and nonsmokers. (A) Cys was lower in smokers (8.7 ± 5.2 μM) than nonsmokers (13.1 ± 5.7 μM). (B) CySS was lower in smokers (86 ± 30 μM) than nonsmokers (99.8 ± 29.3 μM). (C) Total Cys (Cys_t) was lower in smokers (182 ± 62 μM) than nonsmokers (215 ± 57 μM).

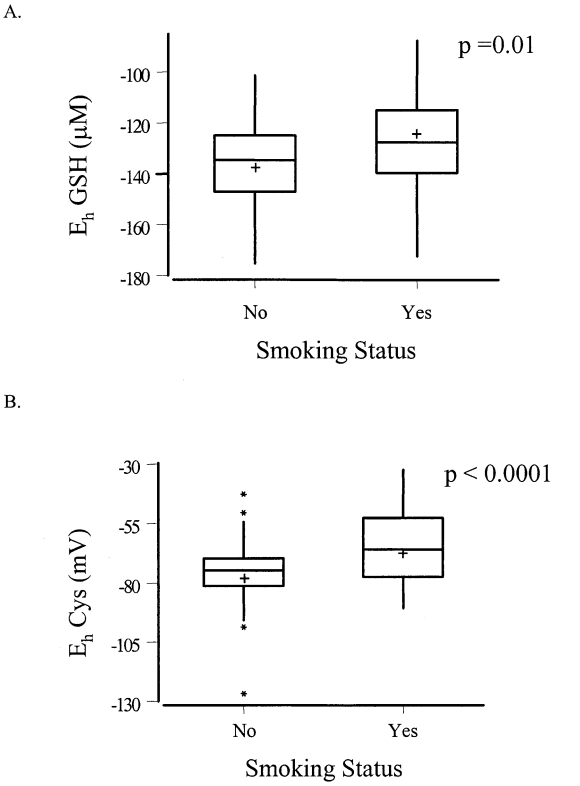

Fig. 3. Comparison of redox states in plasma of smokers and nonsmokers. (A) GSH/GSSG redox couple was oxidized in smokers (−128 ± 18 mV) compared to nonsmokers (−137 ± 17 mV). (B) Cys/CySS redox couple was oxidized in smokers (−64 ± 16 mV) compared to nonsmokers (−76 ± 11 mV).

age, as opposed to the smokers who exhibited a decrease with age. When the metabolite concentrations (other than CySSG) were adjusted for age, the effect of smoking was significant for all of the metabolites except for GSSG, which was not significantly associated with smoking without the age adjustment. Controlling for gender, smoking was significant for Cys, E_h Cys, GSH, CySSG, and GSH_t.

Consistent with other studies [28], the effect of smoking was different for males and females with CySS, Cys_t, and E_h GSH. For males, CySS and Cys_t were lower in smokers, but for females both parameters were slightly increased in smokers. For E_h GSH, male smokers were much more oxidized, whereas female smokers were only slightly oxidized. When analyses were done controlling for differences in race between smoking and nonsmoking groups, smoking was significant for all of the metabolites except GSSG.

With control for difference in supplement use, all the metabolites were significant for smoking except GSSG

Table 2. Significance of Associations Between Smoking and Metabolites with Control for Demographic Characteristics

Metabolite[a]	Age	Gender	Race	Supplements
CySS	0.13	I	.006	.03
Cys	<.0001	<.0001	<.0001	<.0001
E_hCys	.0003	.0004	.001	.0007
GSH	.002	.003	.009	.008
GSSG	.92	.77	.56	.29
CySSG	I[b]	<.0001	.002	.0006
Total GSH	.0001	<.0001	.0006	.0002
E_h GSH	.003	I	.05	.09
Total Cys	.003	I	.002	.008

[a] Each metabolite was assessed for the significance of smoking among the groups when controlling for the subject characteristics listed.

[b] I represents an interaction between the subject characteristic and smoking status. The direction of this interaction is described in the text.

and E_h GSH. Yet, when the subjects taking supplements were removed from both the smoking and the nonsmoking populations, smoking was significant for E_h GSH along with all of the other metabolites and redox values, except for GSSG. When this group was age-adjusted, the effect of smoking remained the same. When the data was adjusted for all the characteristics together, smoking was significant for all of the metabolites except GSSG.

DISCUSSION

Smoking is a risk factor for lung cancer, asthma, and emphysema, as well as many other chronic and degenerative diseases affecting organ systems other than the lung (vascular disease, age-related macular degeneration). Injury in the lung can be explained by the direct chemical exposure to cigarette smoke, but effects causing chronic and age-related diseases in other organ systems are likely to be the result of indirect consequences of the exposure. Several studies show that antioxidants such as vitamin C, lutein, zeaxanthin, β-carotene, and vitamin E are lower in smokers [29,30], and such decreases in antioxidants can result in systemic oxidative stress. However, numerous studies have shown that antioxidant supplements provide only a limited protection, at best [31].

The present study shows that smoking is associated with oxidation and decreased concentrations of the endogenous antioxidant, GSH. Although these changes are consistent with a central role of oxidative stress in promotion of chronic disease, the changes are relatively modest in terms of the overall antioxidant and conjugation capacity of GSH-dependent detoxification systems. In contrast, the changes in Cys/CySS redox are profound, as indicated by a comparison of "redox age" of smokers compared to nonsmokers. For instance, oxidation of Cys/CySS redox occurs with a rate of 0.2 mV/y [14], whereas GSH/GSSG oxidation occurs at a rate of 0.7 mV/y after 45 years [15]. Thus, the 12 mV difference in E_h for Cys/CySS between smokers and nonsmokers is equivalent to smokers being 60 years older (12 mV/0.2 mV per year) than the nonsmokers when compared in terms of Cys/CySS redox. In contrast, when calculated based upon the 9 mV difference for GSH/GSSG, smokers only appear to be 13 years older (9 mV/0.7 mV per year).

The consequences of Cys oxidation and decreased Cys availability are only beginning to be understood. Decreased Cys availability alone can cause an extensive oxidation of GSH/GSSG in cells [22] and result in increased expression levels of thioredoxin-1, another key antioxidant and redox-regulating system [32]. Cells depleted of Cys were more oxidized and had lower levels of GSH, but addition of Cys provided a rapid recovery of GSH/GSSG redox and an overshoot in GSH concentrations [22]. Cys is required for diverse immune functions, including antigen processing, phagocytosis, and T lymphocyte proliferation [21,33,34]. Furthermore, when Cys/CySS redox in culture medium was varied systematically over the range found in vivo in human plasma, the rate of cell proliferation at -80 to -150 mV was twice that at 0 mV [22]. Studies with cultured human retinal pigment epithelial cells showed that cells incubated at the more oxidized redox states were more susceptible to apoptosis [24]. Thus, the decreased availability of Cys, associated with an oxidation of Cys/CySS redox state, may be central to the increased systemic risks of chronic disease associated with cigarette smoking. In support of this, supplementation of rats with N-acetylcysteine (NAC), a precursor of L-cysteine, is protective against smoke-related oxidative damage biomarkers such as DNA adducts [35].

In summary, the present study shows that concentrations of important low molecular weight thiols in human plasma are decreased in association with cigarette smoking. Furthermore, the redox states of both GSH/GSSG and Cys/CySS pools are more oxidized in smokers compared to nonsmokers. The magnitude of Cys/CySS oxidation compared to controls and previous studies of Cys/CySS oxidation with aging indicate that Cys/CySS may be an important parameter in determining systemic effects of smoking. Together with accumulating information concerning the central role of Cys/CySS redox in control of cellular proliferation and apoptosis, these results indicate that perturbations in thiol/disulfide redox could be a central mechanism in the promotion of chronic disease by smoking. If so, nutritional supplementation or other therapeutic interventions designed to improve Cys homeostasis and related Cys/CySS and GSH/GSSG redox could provide a useful interventional strategy.

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ABBREVIATIONS

Cys—cysteine
CySS—cystine
CySSG—mixed disulfide of cysteine and glutathione
Cys_t—total cysteine plus disulfide forms
E_h—redox state
γ-Glu-Glu—γ-glutamylglutamate
GSH—glutathione
GSH_t—total glutathione plus disulfide forms
GSSG—glutathione disulfide
ROS—reactive oxygen species