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Erythrocyte Antioxidant Defense Response Against Cigarette Smoking in Humans—the Glutathione S-Transferase Vulnerability

Hilmi Orhan,¹ Chris T. A. Evelo,² and Gönül Şahin¹

¹Department of Toxicology, Faculty of Pharmacy, Hacettepe University, 06100 Ankara, Turkey

²Department of Pharmacology and Toxicology, Universiteit Maastricht, P.O. Box 616, 6200 MD Maastricht, The Netherlands

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ABSTRACT: Cigarette smoking leads to uptake of a multitude of reactive chemicals including many electrophiles and may also give rise to oxidative stress. Human red blood cells are important targets for electrophilic and oxidant foreign compounds. We investigated the oxidative stress in erythrocytes upon cigarette smoking, and the response of antioxidant defense system against it. With this aim, simultaneous determination of erythrocyte superoxide dismutase (SOD), selenium dependent glutathione peroxidase (Se-GPx), catalase (CAT), glutathione S-transferase (GST) activities and plasma levels of thiobarbituric acid reactive substances (TBARS), and the degree of erythrocyte membrane lipid peroxidation (EMLP) were carried out in blood samples of smokers and their controls. Plasma TBARS levels and EMLP in smokers were significantly higher than the control levels ($p < 0.01$ and $p < 0.005$, respectively). SOD activity was diminished in smokers compared to nonsmoker controls ($p < 0.005$). Erythrocyte Se-GPx activity was also found significantly diminished in smokers ($p < 0.005$), while plasma Se-GPx activity was not changed. We observed that erythrocyte CAT activity was not different in smokers compared to nonsmoker controls. We found that the erythrocyte GST activity is significantly lower in young adult smokers (3.03 ± 0.18 U/mg protein; mean $\pm$ SEM; $n = 46$) than in nonsmoking contemporaries (3.98 ± 0.26 U/mg protein; mean $\pm$ SEM; $n = 41$). Together with previously reported data, it can be concluded that the decrease in GST activity leads to extra GST synthesis during erythrocyte proliferation. The same data were also analyzed for the sex differences. The statistically significant differences remained the same between nonsmoker and smoker females. Only EMLP degree and SOD activity were significantly different between nonsmoker and smoker males;

however, when compared the parameters between male and female nonsmokers, GST activity was found to be significantly higher in females than that of males. © 2005 Wiley Periodicals, Inc. *J Biochem Mol Toxicol* 19:226–233, 2005; Published online in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/jbt.20088

KEYWORDS: *In vivo*; Human; Cigarette Smoking; Antioxidant Enzymes; Erythrocyte; Malondialdehyde

INTRODUCTION

Cigarette smoking leads to the uptake of many hazardous compounds. Such compounds or their metabolites may be electrophilic and thereby able to react with biological macromolecules, or they may give rise to oxidative stress by formation of reactive species or the initiation of radical chain reactions [1].

Oxidative stress emerges as a consequence of imbalance between reducing and oxidizing capacity of the cell in favor of the latter. This process, however, is physiologically observed to some extent due to electron leakage from mitochondria during cellular respiration, serologic defense against invading bacteria and other pathogens, and so on. During these events, reactive oxygen species (ROS) and other free radicals are generated. Due to their extreme reactivity, ROS may react with any compound in the neighborhood. To maintain survival, there is an efficient cellular protective system that tries to convert these species into nontoxic compounds. The quantity of ROS determines whether the protective system will overcome or fail; oxidative stress may appear when the generated ROS exceeds the protective capacity of the cell. Those excess amounts of ROS start to attack cellular macromolecules such as lipids, proteins, and DNA. Any change in these molecules' structures ends up with impaired cellular function, structural degradation, and cellular toxicity

Correspondence to: Hilmi Orhan.

Present address of Chris T.A. Evelo: BiGCaT Bioinformatics; Universiteit Maastricht, Maastricht, The Netherlands.
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to some extent depending on the severity of the stress. A number of prevalent and severe disorders, such as atherosclerosis, mutagenesis and cancer in the upper respiratory system, as well as lungs, have been related to ROS and smoking [2–6].

The erythrocyte is at increased risk from oxidative processes for a variety of reasons. It is continually exposed to high oxygen tensions, hemoglobin is susceptible to autooxidation and can function as an oxidase and a peroxidase [7–9], and it is unable to repair damaged components by resynthesis. Therefore, the erythrocyte is completely dependent on the antioxidant defensive components throughout their 120 days of life span. These components build up an efficient defense system, and an important part of this system consists of three major enzymes: superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) [10].

Superoxide dismutase converts superoxide radicals into hydrogen peroxide, which is actually not a radical, however, generates the most reactive species, hydroxyl anion radical under the appropriate conditions. Hydrogen peroxide is degraded to water and oxygen via GPx at physiological concentrations. When hydrogen peroxide concentration increases until toxic levels, CAT also contributes to the metabolism of this substrate.

Glutathione S-transferase (GST) catalyzes the conjugation of reactive electrophiles to glutathione (GSH) and the reduction of lipid hydroperoxides [11]. In this way, they play an important role in the protective mechanisms against these two challenges from exposure to cigarette smoke. Human erythrocyte may contain three GST isoenzymes: rho (ρ), sigma (σ), and theta (θ). Of these, ρ isoenzyme—which belongs to PI class—constitutes 95% of the activity toward the commonly used substrate 1-chloro-2,4-dinitrobenzene (CDNB). GSTs of the PI class are known to be vulnerable to reactive electrophiles [12,13] and conditions involving oxidative stress [14,15]. ROS such as H_2O_2 and HOCl are found to be potent inhibitors of the enzyme [16,17]. It is known that the former occurs in the tar and/or gas phase of cigarette smoke [18,19], and the latter is excessively generated by cigarette smoke in the body [20]. Therefore, erythrocyte GST may also have interactions with electrophiles and/or oxidants in cigarette smoke.

In the present study, we aimed to investigate the effect of cigarette smoke on human erythrocyte antioxidant defense system in vivo. Therefore, activities of the above-mentioned enzymes have been evaluated in regular smokers and in their control counterparts. To show the degree of cellular membrane lipid damage, blood plasma malondialdehyde (MDA) concentration, which is a secondary end product of membrane lipid peroxidation, has also been analyzed. Besides evaluating the

oxidative damage occurred in RBCs, we also aimed to investigate the tendency of erythrocytes from smokers for degradation by oxidative damage. Therefore, packed erythrocytes of the smokers and the controls were oxidized in an in vitro system by H_2O_2 , and membrane peroxidative degradation was evaluated spectrophotometrically.

MATERIALS AND METHODS

Chemicals

All chemicals used in this study were of analytical grade and purchased from Sigma and Merck Co. GSH, CDNB, NADPH, glutathione reductase, bovine serum albumin, folin phenol reagent, tetraethoxypropane, and Tris were purchased from SIGMA. *n*-Butanol and pyrogallol were obtained from Merck.

Subjects

Seventeen smokers (11 men, 6 women, 32 ± 2.3 mean age) and 17 nonsmokers (8 men, 9 women, 29 ± 2.0 mean age) were studied except for GST activity; 46 smokers (29 men, 17 women, 29 ± 2.2 mean age) and 41 nonsmoker control subjects (26 men, 15 women, 27 ± 2.7 mean age) were studied for erythrocyte GST activity. The study population consisted of graduate students and staff at the university. The blood samples were collected in the morning hours after breakfast and after first cigarettes. None of the subjects were taking any medication. All subjects provided with written informed consent. The ethical standards described by the Helsinki Declaration were followed in the course of the study. For the homogeneity of the study, the smokers were chosen among the individuals who consume 20 cigarettes per day. None of the nonsmokers were exposed second-hand cigarette smoke, since smoking is prohibited in public areas, and there are separate places for smokers in the city as well as in the university.

Blood Collection and Erythrocyte Lysate Preparation

The blood samples were obtained from G6PDH-positive and normal healthy donors to avoid enzyme deficiency caused hemolysis during the experiments. Five milliliter of blood samples was drawn by venopuncture into heparinized tubes. Subsequently, blood samples were centrifuged for 10 min at $4000 \times g$. Plasma samples were separated for the analyses. Erythrocyte lysates were prepared and processed as described previously [21,22]. After removal of plasma

and buffy coats, the red cells were washed twice with two volumes of phosphate buffered saline of pH 7.00. Hemolysates were prepared by addition of two volumes of ice-cold distilled deionized water to the erythrocytes. Cellular debris was removed by centrifugation at $4000 \times g$ and 4°C for 30 min.

Analyses

Superoxide Dismutase

CuZn-SOD activity in erythrocyte lysate was determined according to Marklund and Marklund [23]. Briefly, each hemolysate was further diluted 1:5 with Tris-HCl buffer (pH 8.2; containing 1.2 mM EDTA). Twenty microliter of diluted hemolysate was mixed with 2900 μL of buffer solution in a quartz cuvette. Subsequently, 100 μL of pyrogallol solution (6 mM, in 10 mM HCl) was added to the mixture, mixed for 20 s, and decrease in absorbance was followed at 420 nm for 2 min against buffer solution in a double beam spectrophotometer. Nonenzymatic reaction rate as blank was determined by substituting buffer solution for the sample, and this reaction rate was used for the activity measurements.

Selenium-Dependent Glutathione Peroxidase

Se-GPx activity was determined according to Pleban et al. [24] in both plasma and erythrocyte lysate after dilution of the hemolysate with four volumes of double-strength Drabkin solution. The reaction mixture was 50 mmol/L Tris buffer, pH 7.6 containing 1 mmol/L of Na_2EDTA , 2 mmol/L of reduced glutathione (GSH), 0.2 mmol/L of NADPH, 4 mmol/L of sodium azide, and 1000 U of glutathione reductase (GR). Fifty microliter of plasma and 950 μL of reaction mixture, or 20 μL of plasma and 980 μL of reaction mixture were mixed and incubated for 5 min at 37°C . Then the reaction was initiated with 8.8 mmol/L H_2O_2 , and the decrease in NADPH absorbance was followed at 340 nm for 3 min. Nonenzymatic reaction rate (blank) was determined by substituting nanopure water for the hemolysate or plasma, and this reaction rate was subtracted from the each run. Enzyme activities were reported in U/mg protein in erythrocyte lysate and in U/L in plasma.

Catalase

CAT activity in erythrocyte lysate was measured by the method of Aebi [25] after dilution of the RBC hemolysates 1:500 with 50 mM phosphate buffer, pH 7.00, just before the measurements. The reaction mixture was 50 mM phosphate buffer pH 7.00, 10 mM H_2O_2 , and erythrocyte lysate. The reduction rate of

H_2O_2 was followed at 240 nm for 30 s at room temperature. CAT activity was expressed in U/mg protein.

Glutathione S-transferase

The measurement of GST enzyme activity was performed with 1 mM GSH as the first, and 1 mM CDNB as the electrophilic second substrate in 100 mM potassium phosphate buffer, pH 6.50, according to Habig et al. [26]. One unit of the enzyme activity catalyzed the formation of 1 μmol S-2,4-dinitrophenylglutathione min^{-1} (mg protein) $^{-1}$.

Thiobarbituric Acid Reactive Substance

TBARS test for the MDA content of the plasma samples was performed using the method described by Richard et al. [27]. After the reaction of thiobarbituric acid with malondialdehyde (MDA), the product was extracted in butanol and was measured spectrofluorometrically (excitation: 532 nm, emission 553 nm, slit 10 mm). TBARS levels of plasma were expressed as nmol/mL.

H_2O_2 Forced Erythrocytic Membrane Lipid Peroxidation (EMLP)

For determination of the effect of NSAIDs on H_2O_2 forced lipid peroxidation of membrane, the method of Gutteridge et al. [28] was used. 0.1 mL of packed erythrocytes was added to 0.8 mL of phosphate saline azide (3.4 mM Na_2HPO_4 - NaH_2PO_4 buffer (pH 7.4) in 0.15 M NaCl/7.8 mM sodium azide) and incubated in a shaking water bath at 37°C for 30 min to allow the azide to inactivate catalase within the cells. After adding 0.8 mL of 10 mM H_2O_2 (in 0.15 M NaCl), the cells were incubated for a further 2 h at the same conditions. The reaction was terminated with adding 1 mL of 28% TCA (w/v). All tubes were centrifuged at $4000 \times g$ for 10 min, and 1 mL of the supernatant was collected. One milliliter of TBA 1% (w/v; in 0.05M NaOH) was added to the portion of supernatant, and mixture was heated at 100°C for 15 min. On cooling, 0.1 mL triton X-100 1% (w/v) was added and A_{532} was measured.

Protein

The protein content of the samples was determined according to the method of Lowry modified by Miller et al. [29].

Statistics

All assays were performed in triplicate, and the data are presented as mean values $\pm$ SEM. Statistical

analyses of the data were performed by Student's *t*-test. A probability value of $P < 0.05$ was considered to denote a statistically significant difference.

RESULTS

Figure 1 presents the total erythrocyte and plasma antioxidant parameters of smoker and nonsmoker individuals of the present study. Plasma TBARS levels in smokers are significantly higher than the control levels ($p < 0.01$). EMLP has also been found significantly increased in smokers compared to nonsmoker controls ($p < 0.005$). Erythrocyte antioxidant enzyme activities were affected due to smoking. SOD activity was diminished in smokers compared to nonsmoker controls ($p < 0.005$). Erythrocyte Se-GPx activity was also found significantly diminished in smokers ($p < 0.005$), while plasma Se-GPx activity was not different. We observed

that erythrocyte CAT activity was not different in smokers compared to nonsmoker controls. Glutathione S-transferase activity of smokers has been found significantly lower than that of nonsmoker controls ($p < 0.01$).

The statistically significant differences in parameters remained the same when the data were expressed only for females (Figure 2). Especially the difference between smoker and nonsmoker females' erythrocyte GST activity was pronounced compared to total smokers ($p < 0.005$ vs. $p < 0.01$). The other parameters, plasma TBARS, EMLP, erythrocyte GPx, and erythrocyte SOD were different with the same p value of smaller than 0.05.

Figure 3 summarizes the statistically significant differences between parameters in male smoker and nonsmoker groups. The mean level of erythrocyte membrane lipid peroxidation was found to be significantly higher in smokers compared to nonsmokers, as expected ($p < 0.005$). In contrast, erythrocyte SOD activity

FIGURE 1. Erythrocyte and plasma antioxidant parameters of smoker and nonsmoker individuals. Results are expressed as mean $\pm$ SEM ($n = 41$ for nonsmoker GST analysis, and $n = 46$ for smoker GST analyses, $n = 17$ for all the others). Key: (*) significantly different ($p < 0.01$) from the corresponding control value, and (**) significantly different ($p < 0.005$) from the corresponding control value. EMLP, erythrocyte membrane lipid peroxidation.

FIGURE 2. Erythrocyte and plasma antioxidant parameters of female smoker and nonsmoker individuals. Results are expressed as mean $\pm$ SEM ($n=15$ for nonsmoker and $n=17$ for smoker GST analyses, $n=9$ nonsmoker and $n=6$ smoker for all the others). Key: (#) significantly different ($p < 0.05$) from the corresponding control value, and (**) significantly different ($p < 0.005$) from the corresponding control value. EMLP, erythrocyte membrane lipid peroxidation.

was lower in smoker males compared to nonsmoker males ($p < 0.05$).

Among the parameters, there was a statistically significant difference in erythrocyte GST activity between female and male nonsmokers (Figure 4, $p < 0.005$). The other insignificant different parameters were not shown in Figures 2–4.

DISCUSSION

The main objective of the present study was to evaluate the toxic outcomes of cigarette smoking on erythrocytes and investigate the status of antioxidant defense enzymes toward the deleterious effects of smoking. Our results clearly show that smoking induces oxidative

FIGURE 3. Erythrocyte and plasma antioxidant parameters of male smoker and nonsmoker individuals. Results are expressed as mean $\pm$ SEM ($n=26$ for nonsmoker and $n=29$ for smoker GST analyses, $n=8$ nonsmoker and $n=11$ smoker for all the others). Key: (#) significantly different ($p < 0.05$) from the corresponding control value, and (**) significantly different ($p < 0.005$) from the corresponding control value. EMLP, erythrocyte membrane lipid peroxidation.

FIGURE 4. Erythrocyte glutathione S-transferase activity of male and female nonsmoker individuals. Results are expressed as mean $\pm$ SEM ($n = 26$ males and $n = 15$ females). Key: (**) significantly different ($p < 0.005$).

lipid degradation in smokers, as shown by increased plasma TBARS levels (Figure 1). This result is in accordance with previously published reports [30–32]. It shows that erythrocytes cannot cope with the oxidative stress caused by consuming approximately 20 cigarettes a day, and oxidative damage to membrane lipids takes place. As a secondary effect, smoking increased the tendency of oxidative degradation of erythrocyte membrane. It is well known that increase in TBARS levels indicates that there is a potential risk for pathologies. On the other hand, it is not possible to make quantitative assessments between blood TBARS levels and risk of pathologies, since there are not adequate epidemiological data.

Tungtrongchitr and coworkers [33] reported a higher incidence of anemia in smokers compared to nonsmokers; however, they did not relate this observation to serum folic acid levels in smokers, as it was not statistically lower than that of nonsmokers levels. In another study, the erythrocyte folate level, which is more reliable indicator of blood folate levels compared to serum, was also not different in smokers compared to nonsmokers [34]. Therefore, our present observation that erythrocyte membrane of smokers is more readily oxidized may explain, at least partially, the anemia disorder usually associated with smoking.

Antioxidant enzyme responses against smoking varied in the present study. The two of the main antioxidant enzymes, SOD and GPx activities in erythrocytes, were significantly lower in smokers compared to nonsmokers. However, plasma GPx and erythrocyte CAT activities were found not to be affected by smoking. Superoxide dismutase uses superoxide anion radical as substrate and converts it into H_2O_2 . Subsequently, H_2O_2 is converted to H_2O by GPx at physiological concentrations. Catalase acts in the same way as GPx; however, this enzyme contributes when the concentration of the substrate (H_2O_2) is highly increased. Thus, SOD and GPx are the first enzymes that confront and hold with the reactive substrates. This may explain why these two enzyme activities were diminished upon smoking.

When the data were expressed only for female smokers, the decrease in erythrocyte GST activity was more pronounced compared to total smokers. On the other hand, the basal GST activity was significantly higher in nonsmoker females compared to nonsmoker males. This suggested that GST in females is more vulnerable to oxidative stress caused by smoking.

The other statistically significant differences were not changed between smoker and nonsmoker females. However, only EMLP and SOD were significantly different in smoker and nonsmoker males. Again, this suggests that females are in general more susceptible to oxidative stress caused by smoking.

It has been previously demonstrated that GST is vulnerable to oxidative stress [14,15], for which the reason has been attributed to the presence of cysteine moieties at position 14, 47, 101 and 169 from the N-terminus are involved in this inhibition. The most reactive group is Cys47 [35,36]. It is easily conceivable that cigarette smoking may also influence erythrocyte GST activity, as it contains or cause to generate at least two potent erythrocyte GST P inhibitors, H_2O_2 and HOCl either in the tar or gas phase [18–20].

Glutathione S-transferase activities of 3.03 ± 0.18 U/mg protein (mean $\pm$ SEM) were found for the 46 smokers in the present study; this value is significantly lower than what was found for the 41 nonsmoker controls (3.98 ± 0.26 U/mg protein). This statistically significant lower value in smokers is in accordance with the study published earlier [37], where the same phenomenon was seen in a group of smokers from The Netherlands. In that study, strongly elevated GST PI protein concentrations in the smokers were found using an ELISA technique. This seems to indicate that the decrease in GST activity—for that some confirmation is given in this study—leads to extra GST synthesis during erythrocyte proliferation. Further comprehensive studies are still needed to clarify the effect of smoking on erythrocyte GST activity and induction of enzyme synthesis.

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