ASCORBIC ACID RECYCLING IN HUMAN ERYTHROCYTES IS INDUCED BY SMOKING IN VIVO

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Abstract—Tobacco smoke contains large numbers of radicals that burden the antioxidant defense and, thus, lower plasma antioxidants, in particular vitamin C or ascorbic acid, is commonly observed among smokers. Ascorbic acid recycling describes the process in which ascorbic acid is oxidized to dehydroascorbic acid by various pathways and subsequently reduced back to ascorbic acid intracellularly, e.g., in erythrocytes, thereby preserving the ascorbic acid pool. In humans who are unable to synthesize ascorbic acid, and in smokers in particular, who are prone to oxidation, this process must be very efficient and of great importance. It has previously been reported that isolated erythrocytes subjected to tobacco smoke in vitro had significantly lower ascorbic acid recycling as compared to controls. In contrast to these findings, we now report that freshly isolated erythrocytes from long-term smokers (n = 39) display a significantly increased rate of ascorbic acid recycling in vivo as compared to those isolated from nonsmokers (n = 31; p < .0001). Preliminary data suggests that the increase results from induction of dehydroascorbic acid reductase activity rather than from differences in energy status, glutathione content, or altered transport capacity. The induction of ascorbic acid recycling as a potential adaptation mechanism of the antioxidant defense to oxidative insults is discussed.

Keywords—Ascorbic acid, Ascorbic acid recycling, Dehydroascorbic acid, Smoking, Clinical trial, Dehydroascorbic acid reductases, Glutathione, Glutathione reductase, Free radicals

INTRODUCTION

Smoking is associated with lower plasma antioxidants concentrations, increased oxidative stress and damage, and increased risk of several chronic diseases [1–7]. Specifically, depletion of vitamin C, or ascorbic acid (AA), has long been a known effect of cigarette smoking [8]. The depletion probably originates from at least two different effects; one of diet and one of smoking per se. Regarding diet, several studies have reported that smokers consume fewer micronutrients than nonsmokers, thereby contributing to lower levels of several antioxidants [9–11]. However, we have recently shown that AA is depleted by smoking per se in a study with matched dietary intakes of antioxidants [12]. The latter correlates well with the high number of radicals known to be contained in tobacco smoke [5,13]. Moreover, while smoking cessation quickly results in about 25% increased plasma ascorbate [14], the level in ex-smokers may remain lower than that of never-smokers for years [15]. This supports a biphasic depletion/repletion process. In spite of these observational advances, the molecular basis for the effects of smoking on plasma AA remains unclear.

When AA acts as a radical quencher in vivo it is oxidized via intermediate radicals to dehydroascorbic acid (DHA). DHA is a labile compound and is rapidly and irreversibly hydrolyzed in vitro with a half-life of only a few minutes at physiological pH [16]. To prevent this unfavorable event in vivo, DHA is efficiently reduced back to AA intracellularly, e.g., by erythrocytes, hepatocytes, and other cells. This process is commonly referred to as ascorbate recycling [17,18]. Consequently, recycling of AA plays a key role in preserving its antioxidant function. In humans, where relatively small
amounts of vitamin C are required from the diet to prevent pathologies like scurvy, this process must be very efficient and of great importance. May and coworkers have pioneered the investigation of ascorbate recycling in erythrocytes with a series of studies [17,19–27]. They have demonstrated that recycling of AA in erythrocytes occurs predominantly via glutathione (GSH)-dependent DHA reductases, apparently with a small contribution from reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent DHA reductases such as thioredoxin reductase.

In healthy nonsmoking humans, the plasma concentration of DHA is close to nil [28,29], indicating the high efficiency of the recycling process. However, in a cross-sectional population study, we have shown specifically that smokers with low plasma concentrations of AA have an elevated level of plasma DHA [8]. This phenomenon was not found among the respective nonsmokers in whom no DHA was present regardless of their AA concentration [8]. The reason for an increased presence of plasma DHA among smokers with low AA might be the relatively larger oxidant load per antioxidant molecule in these subjects. Alternatively, it could indicate that the AA recycling process in smokers is somehow impaired.

In contrast to the latter explanation, we recently discovered that enzymatic recycling of AA is induced by severe oxidative stress in a guinea pig model, presumably as a protective measure [30]. Guinea pigs, like humans, lack the ability to synthesize AA and, therefore, rely entirely on dietary supplementation. Based on these new results, we hypothesized that AA recycling might also be induced by smoking in humans. This could represent a hitherto undiscovered secondary compensatory response in the human antioxidant defense.

In the present article, we report the results from a study in which the effect of smoking on the recycling of AA in erythrocytes was examined in a cohort of smoking and nonsmoking men.

**EXPERIMENTAL PROCEDURES**

**Subjects**

The study was conducted in accordance with the Declaration of Helsinki and approved by the Local Ethics Committee of Copenhagen. Seventy healthy male volunteers (39 smokers and 31 nonsmokers) between 21 and 63 years of age were recruited from the greater Copenhagen area using newspaper advertisement. Inclusion criteria were as follows: >18 years of age, smoking more than 5 cigarettes per day (smokers only), normal weight [20 kg/m² ≤ body mass index (BMI) ≤ 30 kg/m²], and signing the informed consent form. Exclusion criteria were chronic diseases, regular use of medicine, any use of vitamin or other dietary supplements after prescreening, and smoking within the last 2 years (nonsmokers only). Information on any previous use of vitamin supplements was recorded. No subjects withdrew from the study after recruitment.

An overnight fasting blood sample was collected from each subject by using ethylenediaminetetraacetic acid (EDTA) as anticoagulant. The blood samples were immediately centrifuged at 2000 × g for 5 min (4°C) and erythrocytes and plasma were separated and processed for the following analyses as indicated below.

**Biochemical analyses**

Plasma samples for AA measurement were immediately stabilized with an equal amount of 10% (wt:vol) meta-phosphoric acid containing 2 mM of disodium-EDTA. The precipitate was removed by centrifugation at 16,000 × g for 1 min (4°C) and the supernatants were stored at −80°C for less than 1 month until analysis. The concentration of AA in plasma was measured by using reversed-phase high-performance liquid chromatography (HPLC) with coulometric detection as described elsewhere [31,32]. Erythrocyte GSH was measured before and after the recycling experiments by the method of Hissin and Hilf [33]. In the calculation of intracellular concentrations, erythrocyte cytoplasm was taken as 70% of the packed cell volume [34]. Hemoglobin was measured by spectrophotometry after conversion to methemoglobin with ferricyanide using a commercial kit (Sigma, St. Louis, MO, USA). GSH reductase activity was estimated by the method of Anden et al. [35]. Plasma malondialdehyde (MDA) was measured as its dithiobarbituric acid adduct by HPLC with fluorescence detection as described previously [36].

**AA recycling**

AA oxidation is the rate limiting step in AA recycling, so in pursuing differences in recycling capacity, DHA reductase activity have to be measured directly. Consequently, AA recycling in erythrocytes was estimated by measuring the rate of DHA reductase [37,38], i.e., the combined activity of enzymes capable of reducing DHA to AA. Erythrocytes were washed three times with 5 volumes of phosphate-buffered saline (PBS) followed by centrifugation at 2000 × g for 5 min (4°C). With each wash, the “buffy coat” layer of white cells was carefully removed to prevent contamination. After the final wash, the hematocrit was adjusted to 25% with PBS and aliquots were frozen at −80°C for GSH reductase activity measurement or used immediately for recycling experiments essentially as described by May and coworkers [26] and by Lykkesfeldt [30]. Briefly, erythrocytes were adjusted to a final 6% hematocrit with PBS. The assay
involved a 20 min incubation period at 37°C with 100 μM DHA followed by HPLC analysis of the intracellularly accumulated AA as described elsewhere [38]. The basic assay was performed after a 20 min preincubation at 37°C in the presence and absence of 5 mM D-glucose (GLU). Pilot experiments (data not shown) revealed that under these conditions, DHA absorption is virtually complete after the 20 min incubation period, while about 50% of the added DHA is left unreduced intracellularly. This suggests that DHA uptake is not rate-limiting the present experimental setup. AA was not detectable extracellularly, indicating that diffusion does influence the measurement of AA accumulation, i.e., the rate of DHA reduction. Moreover, observed differences in rate of AA accumulation persisted throughout the time course.

The AA recycling process was further characterized by performing parallel recycling experiments with erythrocytes after preincubation with various assay inhibitors. Thus, recycling experiments were carried out following a 20 min preincubation at 37°C with (i) 2 mM tert-butylhydroperoxide (TBH), an oxidant known to induce lipid peroxidation and deplete GSH [39], (ii) 0.5 mM 1-chloro-2,4-dinitrobenzene (CDNB), and (iii) 5 mM phorone (PHO), which deplete GSH by glutathione-S-transferase-dependent conjugation [26,40,41], or (iv) 0.1 mM phenylarsine oxide (PAO), an agent that reacts with vicinal thiols [42], but also inhibits the DHA reductase thioredoxin reductase [26,40,41], or (iv) 0.1 mM phenylarsine oxide (PAO), an agent that reacts with vicinal thiols [42], but also inhibits the DHA reductase thioredoxin reductase [26]. All the latter experiments were performed in the presence of 5 mM GLU. The compounds were dissolved and added in dimethylsulfoxide. The final concentration of dimethylsulfoxide was 0.5%; a concentration that alone in parallel experiments showed no interference with the recycling process (data not shown).

Statistical analysis

Data were analyzed by using Statistica 6 (StatSoft, Tulsa, OK, USA). Homogeneity of variances was verified by Levene’s test. Differences between smokers and nonsmokers were tested by repeated measures analysis of variance (ANOVA) followed by post-hoc t-tests. Effects of recycling inhibitors were tested by using paired t-test. A two-tailed p value < .05 was considered statistically significant. Values are reported as mean ± SD.

RESULTS

Baseline measurements of the study population

The population sample was initially characterized by the variables listed in Table 1. Surprisingly, there was no difference in plasma AA level between smokers and nonsmokers in the present cohort. The effect of smoking on plasma AA has been documented on numerous other occasions [15,43–45]. For unknown reasons, the nonsmokers’ average plasma AA in the present study is markedly lower compared with our previous studies [8,46] and resembles that of a population with a low intake of fruit and vegetables, i.e., more comparable to the smokers group [12]. This indicates that the nonsmokers included in the present study do not represent the average population. However, although unintentional in the present case, this has little impact on the present study and actually further enables the isolation of the effect of smoking per se. Smokers had similar erythrocyte GSH as nonsmokers while a tendency towards lower GSH reductase activity compared to nonsmokers was observed (p = .13).

Effect of smoking on AA recycling in erythrocytes

Two-way ANOVA showed a significant effect of smoking on the recycling of AA (p < .0001). This effect persisted in the unchallenged experiments (p < .01 or p < .001, Fig. 1). Addition of 5 mM glucose to the basic reaction mixtures increased AA recycling by about 100% in both smokers and nonsmokers (p < .001, Fig. 1). This suggests that difference in energy status does not account for the higher recycling of smokers. In a pilot experiment using blood samples from 10 individuals, recycling experiments using lysed erythrocytes were carried out in parallel. The difference in AA recycling between smokers and nonsmokers was equivalent regardless of whether lysed or intact erythrocytes were used, indicating that differences in DHA transport capacity do not play a role in the observed effect of smoking (data not shown). To further demonstrate the involvement of enzymes in the recycling of AA in erythrocytes, GSH was measured before and after the incubations and a variety of challenges were presented to the recycling reaction system (Figs. 1 and 2).

The role of GSH in AA recycling

As mentioned above, GSH was not higher in smokers compared with nonsmokers when initially measured (Ta-

| Table 1. Baseline Measurements of the Study Population |
|-------------|----------|----------|
|            | Nonsmokers | Smokers  |
| Age (y)    | 35.6 ± 12.3| 37.3 ± 11.7|
| Body weight (kg) | 79.8 ± 9.7  | 76.2 ± 11.9 |
| Body mass index (kg/m²) | 24.3 ± 2.6  | 23.5 ± 2.8  |
| Cigarettes per day | 16.8 ± 8.2 | 16.8 ± 8.2 |
| Pack-years (y) | 15.1 ± 13.3 | 15.1 ± 13.3 |
| Plasma ascorbic acid (μM) | 45.8 ± 16.2 | 45.2 ± 17.9 |
| Erythrocyte glutathione (mM) | 2.5 ± 0.3 | 2.4 ± 0.4 |
| Erythrocyte glutathione reductase activity (μmol NADPH oxidized/min/mg Hgb) | 5.0 ± 0.5 | 4.8 ± 0.6 |

Values are mean ± SD. NADPH = reduced nicotinamide adenine dinucleotide phosphate.
Challenges were presented to the system by preincubating with 2 mM tert-butylhydroperoxide (TBH), 0.5 mM 1-chloro-2,4-dinitrobenezene (CDNB), 5 mM phorone (+PHO), or 0.1 mM phenylarsine oxide (+PAO), all dissolved in dimethylsulfoxide (0.5% final concentration) and in the presence of 5 mM t-glucose. Following the incubation period, the reactions were quenched and aliquots subjected to high-performance liquid chromatography analysis for accumulated ascorbic acid and glutathione (Fig. 2). Error bars are standard deviations. **p < .001, ***p < .0001 compared to nonsmokers in respective experiment by one-way analysis of variance (ANOVA). Two-way ANOVA showed a significant effect of smoking on the recycling of AA (p < .0001).

Fig. 1. Effect of smoking on ascorbic acid recycling in erythrocytes. Recycling of ascorbic acid in intact erythrocytes isolated from smokers (n = 39, closed bars) and nonsmokers (n = 31, open bars). Erythrocytes diluted with phosphate-buffered saline to 6% hematocrit were incubated 20 min at 37°C with gentle rocking (8 rpm) in the presence of 100 μM dehydroascorbic acid following a 20 min preincubation in the absence (−GLU) and presence (+GLU) of 5 mM t-glucose. Challenges were presented to the system by preincubating with 2 mM tert-butylhydroperoxide (+TBH), 0.5 mM 1-chloro-2,4-dinitrobenezene (+CDNB), 5 mM phorone (+PHO), or 0.1 mM phenylarsine oxide (+PAO), all dissolved in dimethylsulfoxide (0.5% final concentration) and in the presence of 5 mM t-glucose. Following the incubation period, the reactions were quenched and aliquots subjected to high-performance liquid chromatography analysis for accumulated ascorbic acid and glutathione (Fig. 2). Error bars are standard deviations. **p < .01, ***p < .001 compared to nonsmokers in respective experiment by one-way analysis of variance (ANOVA). Two-way ANOVA showed a significant effect of smoking on the recycling of AA (p < .0001).

Table 1). In the recycling experiments, smokers were also found to have similar concentrations as nonsmokers. This demonstrates that the increased recycling observed in smokers is not due to increased GSH, as might have been suspected if AA recycling was merely a chemical reduction by GSH (Fig. 2). The consumption of GSH during the basic recycling experiment (calculated by subtracting the individual end-values from the corresponding start-values) was 40% larger among the smokers, although this increase did not reach statistical significance. However, ANOVA showed a significant effect of smoking on GSH consumption in general (p < .005). This corresponds well to the increased recycling rate among smokers, thus suggesting that GSH expenditure was directly linked to AA recycling.

The inclusion of various oxidants or inhibitors was aimed at further clarifying the role of GSH in AA recycling in erythrocytes as well as detecting possible differences in susceptibility of smokers and nonsmokers to these challenges (Figs. 1 and 2). Overall, no effects of smoking were observed in these experiments, i.e., the ratios between smokers and nonsmokers remained virtually unchanged. TBH (2 mM) completely abolished the AA recycling activity (p < .001) while depleting intracellular GSH by 90% (p < .001). A similar effect was obtained with CDNB (p < .001). PHO (5 mM) resulted in a 20% decrease in AA recycling (p < .001) and was accompanied by a 22% drop in intracellular GSH level (p < .001). TBH and PHO are known to deplete GSH relative selectively by oxidation [39] or glutathione-S-transferase-dependent conjugation [40,41] and our results therefore apparently support an approximate dose-dependent consumption of GSH in the recycling of AA in erythrocytes. In addition to GSH depletion via glutathione-S-transferase-dependent mechanisms, CDNB has also been shown to be an inhibitor of mammalian thioredoxin reductase [47,48], a NADPH-dependent DHA reductase. In contrast to the above data, the inclusion of PAO in the reaction mixture resulted in only a 55% reduction of erythrocyte GSH (p < .001), while 98% of the AA recycling activity disappeared (p < .001). PAO reacts with vicinal sulfhydryl groups and has also been reported to inhibit enzymes involved in the reduction of DHA [26].

**AA status, oxidative stress, and erythrocyte recycling**

Our previous studies have suggested that depletion of AA or increased oxidative stress could potentially be inducers of AA recycling [30]. Figure 3 shows plasma AA vs. AA recycling in erythrocytes. No correlation (y = 0.02361 x + 39.7; r² = 0.0009; p = .81) between AA concentration and recycling was found indicating that low plasma AA alone does not induce AA recycling. In contrast, as shown in Fig. 4, in the case of plasma MDA vs. AA recycling in erythrocytes a significant positive correlation (y = 19.6 x + 22.7; r² = 0.13; p = .002) was found suggesting that increased oxidative stress may induce AA recycling in human erythrocytes.

**No effect of previous vitamin supplement use among smokers**

Any use of vitamin supplements prior to the prescreening of the subject was recorded because supplementation could potentially influence the recycling of AA if antioxidant status is involved in the regulation of this activity. Figure 5 shows recycling activities among smokers, depending on whether a previous use of vitamin supplements was recorded. In the basic recycling experiment without glucose (−GLU), a positive effect of supplementation was observed on AA recycling (p < .05), while the remaining experiments showed no significant effects. Repeated measures ANOVA showed a trend for supplementation as a general positive effect (p = .07). Those subjects who took vitamin supplements were asked to discontinue at the prescreening, and 4 weeks later, when the study began, there was no difference in plasma AA between supplement users and con-
trols (45.4 ± 20.5 and 45.1 ± 16.7 μM, respectively). The results further substantiates that no direct correlation between AA recycling and antioxidant status could be observed.

DISCUSSION

Recycling of AA plays a key role in its antioxidant function. In smokers that are prone to poorer diet and supposedly increased oxidative stress compared with nonsmokers, this process must be of even greater importance. In a previous study, we found that while nonsmokers had no DHA in their plasma regardless of their AA status, plasma DHA in smokers was inversely correlated with their plasma AA concentration [8]. This led us to suggest that maybe AA recycling is partially impaired in smokers. The notion was supported by a recent study, in
which it was shown that isolated human erythrocytes exposed to cigarette smoke in vitro have decreased AA recycling [49]. In the present study, the in vivo effect of smoking on the rate of AA recycling was assessed by incubating freshly isolated erythrocytes from smokers and nonsmokers with DHA, i.e., measuring DHA reductase activity as an index of AA recycling. In order to be able to discriminate between differences in DHA transport ability (mainly GLUT1 transport) and DHA reductase activity, pilot experiments were carried out using lysed and intact erythrocytes in parallel.

Our results show that AA recycling is increased rather than attenuated by smoking in vivo (Fig. 1). The increase in recycling rate among smokers was not accompanied by an increased GSH concentration (Table 1). Indirectly increased GSH capacity could arise from, e.g., higher GSH reductase activity among the smokers. However, there was no significant difference in GSH reductase activity of erythrocytes between smoker and nonsmokers (Table 1). Addition of 5 mM GLU to the assays increased AA recycling by about 100% in both the smokers and nonsmokers, suggesting that differences in energy status did not participate in the observed effect of smoking. Moreover, there was no significant difference between experiments carried out with lysed compared with intact erythrocytes. This indicates that DHA transport is not rate-limiting and that changes in transport activity cannot explain the increased recycling among smokers (data not shown). Consequently, our preliminary investigations indirectly suggest that the observed increase in AA recycling rate is due to induced DHA reductase activity.

Human erythrocytes have a lifespan of about 4 months. Erythrocytes of an adult mammal lack a nucleus, endoplasmic reticulum, mitochondria, and ribosomes. Thus, induction of enzymatic activity is not possible in the mature erythrocyte. Reticulocytes may have this ability but they amount to only a minor fraction of the blood cells. Thus, an increase in DHA reductases most likely originates from an altered erythrocyte production in the stem cells of the bone marrow [30]. Consequently, the data indicate that changing the recycling rate of the erythrocytes may constitute a secondary part of the available response to oxidative stress in smokers. The effect can presumably only be observed in long-term smokers, as those included in the present study, because a substantial proportion of the erythrocytes must be replaced in order to increase the overall capacity. This could be a reason for the apparent discrepancy between the results obtained in the present study and those reported above from in vitro studies. Moreover, as the authors used in vitro exposure of erythrocytes to cigarette smoke [49], their experiment has more resemblance with our susceptibility tests, in which cells were preincubated with, e.g., TBH in vitro. In agreement with their findings, our data showed a significant loss of recycling activity after direct exposure to increased oxidative stress.

Incubation with a variety of inhibitors of AA recycling was conducted in order to test if an increased susceptibility due to smoking could be detected and to further characterize the induction of enzymatic activity. As shown in Fig. 1, the ratio of smoking-induced increase in AA recycling was approximately unchanged regardless of the treatment, indicating that smoking did not further increase susceptibility. Pretreatment with TBH or CDNB largely abolished all recycling activity (Fig. 1) by depleting cellular GSH stores similarly (Fig. 2). Inclusion of PHO caused a 20% decrease in recycling activity, which was matched by a similar drop in GSH. These data indicate that GSH is involved in the recycling of AA in erythrocytes and the proportional decrease in AA recycling and GSH may suggest a dose-dependent relationship. However, there was no significant correlation between AA recycling and GSH consumption. In contrast, pretreatment of erythrocytes with PAO resulted in a disappearance of virtually all recycling activity, while only a 55% drop in intracellular GSH was observed. Our results are consistent with those of Mendiratta et al. [26], who concluded that one or more sulfhydryl-containing enzymes, such as glutaredoxin or thioredoxin reductase, are involved in the recycling process.

GSH- and NADPH-dependent DHA reductases have previously been characterized. More specifically, rat liver GSH-dependent DHA reductase has recently been
cloned [50]. May’s group has studied DHA reductase activity of human erythrocytes and concluded that while DHA reduction is largely GSH-dependent, presumably mediated by glutaredoxin, a small contribution from NADPH-dependent enzymes, presumably thioredoxin reductase, is also available [19,24]. Our present results cannot distinguish between GSH- and NADPH-dependent DHA reductase activities. Consequently, whether one or both types of enzymes are upregulated in smokers remains unclear. However, based on the GSH measurements in the present study it would seem most likely that GSH-dependent DHA reductases are induced because the GSH expenditure relative to AA recycling is similar between smokers and nonsmokers. This view is further supported by the significant effect of smoking observed on GSH expenditure by ANOVA. However, further studies are needed, perhaps using aurothioglucose specific inhibition of thioredoxin reductase activity [26], to clarify this point.

The possible link between oxidative stress and erythrocyte recycling activity was reported recently in guinea pigs, which, like humans, are unable to synthesize AA [30]. In young animals, where a substantial proportion of the erythrocytes had been exchanged during a period of severe AA deficiency, AA recycling activity of the erythrocytes was twice that of control animals, in spite of unchanged GSH levels [30]. These data indicated that low AA status or increased oxidative stress may trigger inhibition of thioredoxin reductase activity [26], to clarify this point.

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**ABBREVIATIONS**

AA—Ascorbic acid
CDNB—1-Chloro-2,4-dinitrobenzene
DHA—Dehydroascorbic acid
GLU—Glucose
GSH—Glutathione
MDA—Malondialdehyde
PAO—Phenylarsine oxide
PHO—Phorone
TBH—*tert*-Butylhydroperoxide