

Ascorbate Recycling by Erythrocytes During Aging in Humans

Syed Ibrahim Rizvi, Kanti Bhooshan Pandey, Rashmi Jha, and Pawan Kumar Maurya

Abstract

Erythrocytes play a crucial role in recycling ascorbate in blood plasma. The erythrocyte ascorbate free radical (AFR) reductase is involved in the reduction of AFR to ascorbic acid (ASC) in the plasma. In the present study, we report an age-dependent increase in the activity of erythrocyte AFR reductase in humans that shows a significant positive correlation with the activity of plasma membrane redox system (PMRS). We explain the age-dependent increase in erythrocyte ASC recycling on the basis of a compensatory/protective mechanism that operates to maintain the ASC level in plasma and thereby minimize oxidative stress during aging.

Introduction

ASCORBIC ACID (ASC) IS THE PRIMARY antioxidant present in plasma; however, humans, higher primates, and guinea pigs cannot make ASC and thus require it through the diet. For all other animals, ASC is not a vitamin. In the presence of an oxidant, ASC is oxidized first to monodehydroascorbate or ascorbate free radical (AFR) and then to dehydroascorbate (DHA), which is unstable and undergoes irreversible hydrolysis to 2,3-diketo-L-gulonic acid, resulting in decreased level of the vitamin.^{1,2} Two molecules of AFR can react with each other to form one each of ASC and DHA. In recent years, there has been much interest in the mechanisms by which ASC level is maintained in blood.

Erythrocytes, being the most abundant cells in the blood, have been reported to play a crucial role in recycling ASC in blood plasma.³ Erythrocytes can take up DHA from the plasma through the GLUT1 glucose transporter. Inside the cell, DHA can be recycled to ASC via direct reduction by glutathione,⁴ by glutathione-dependent enzymes such as glutaredoxin and protein-disulfide isomerase,⁵ and by nicotinamide adenine dinucleotide phosphate oxidase (NADPH)-dependent thioredoxin reductase.⁶ Because the rate of release of ASC from erythrocytes to plasma is slow, the role of recycling of ASC in the maintenance of plasma ASC levels assumes importance.

Studies show that human erythrocytes contain a plasma membrane redox system (PMRS) that transfers electrons from intracellular donors (NADH and/or ASC) to extracellular acceptors, although the physiological acceptor is still unclear.⁷ There is evidence that the intracellular ASC donates

electrons to extracellular AFR via the PMRS, which incorporates an AFR reductase.⁸ Such a redox system enables the cells to counteract oxidative processes effectively and thereby prevent depletion of extracellular ASC.⁸ This provides an efficient mechanism for ASC recycling between the intra- and extracellular compartments.⁷

There is overwhelming evidence to show that aging is associated with increased oxidative stress. The plasma antioxidant capacity has been reported to decrease during aging in humans.⁹ We have reported an age-dependent increase in the activity of erythrocyte transmembrane electron transport that has been explained as a compensatory mechanism of the system to maintain plasma antioxidant levels.¹⁰ The present study was undertaken to determine the rate of ascorbate recycling between erythrocyte and plasma, measured in terms of erythrocyte AFR reductase activity, during human aging. We also correlated the activity of erythrocyte AFR reductase with PMRS, as a function of human age, in an effort to understand the role of ASC recycling in human aging.

Materials and Methods

The study was carried out on 61 normal healthy subjects of both sexes between the ages of 22–79 years following the criteria reported earlier.^{9,10} The subjects were screened for diabetes mellitus, asthma, tuberculosis, or any other major illness. None of the subjects were smokers or were taking any medication. All persons gave their informed consent for the use of their blood samples for the study. The protocol of study was in conformity with the guidelines of the Institutional Ethical Committee.

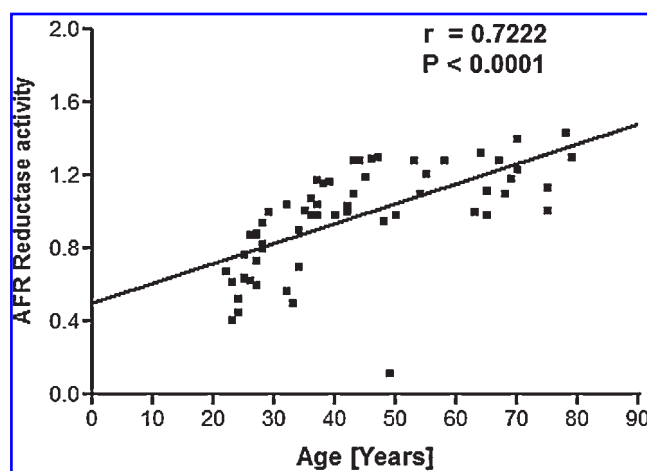


FIG. 1. Erythrocyte AFR reductase activity was determined in 61 blood samples taken from persons of different ages. The plot shows the dependence of AFR reductase activity as a function of human age. AFR reductase activity expressed in terms of μmol NADH oxidized/min per mL of packed red blood cells.

Human venous blood from different healthy volunteers was obtained by venipuncture in heparin. The blood was centrifuged at $1800 \times g$ for 10 min at 4°C . After removal of plasma and buffy coat, the red blood cells (RBCs) were washed twice with cold phosphate-buffered saline (PBS; 0.9% NaCl, 10 mM Na_2HPO_4 , pH 7.4).

The erythrocyte AFR reductase activity was assayed following the method described by May et al.¹¹ The washed erythrocytes were hemolysed and diluted 100% (vol/vol) by addition of water followed by centrifuging for 10 min in the cold. AFR was generated in diluted hemolysates by incubating them at 37°C in PBS (pH 7.0), containing 1 mM ascorbate, 5 units/mL ascorbate oxidase, and 0.1 mM of nicotinamide adenine dinucleotide (NADH). The rate of NADH oxidation was measured spectrophotometrically at 340 nm for 3 min. at 37°C . The change in NADH concentration was calculated from the slope of the resulting line, using an extinction coefficient $\epsilon = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$. The values were corrected in each experiment for the rate observed with lysate and reduced nucleotide alone. AFR reductase activity is reported in terms of μmol NADH oxidized/min per mL of packed red blood cells.

Erythrocyte trans-plasma membrane redox activity was estimated by following the reduction of ferricyanide according to the method of Avron and Shavit.¹² A total of 0.2 mL of RBCs were suspended in PBS containing 5 mM glucose and 1 mM freshly prepared potassium ferricyanide. The suspension was incubated for 30 min at 37°C and then centrifuged at $1800 \times g$ at 4°C . The supernatant collected was assayed for ferrocyanide content using 4,7-diphenyl-1,10 phenanthrolinedisulfonic acid disodium salt and measuring absorbance at 535 nm ($\epsilon = 20,500 \text{ M}^{-1} \text{ cm}^{-1}$). Results are expressed in μmol ferrocyanide/mL of PRBC per 30 min.

Relationships between various parameters were assessed using the Pearson correlation coefficient (r) and coefficient of determination (r^2). Statistical analyses were performed using GraphPad Prism version 4.00 for Windows, GraphPad Software (San Diego CA).

Results

Figure 1 shows the activity of erythrocyte AFR reductase during human aging. We show a significant age-dependent increase in the activity of erythrocyte AFR reductase. In an earlier report, we had already reported a similar age-dependent increase in erythrocyte PMRS activity.¹⁰ To investigate the age-dependent increase in AFR reductase activity and its association with PMRS further, we also determined the PMRS activity in same blood samples. The relationship between erythrocyte AFR reductase and PMRS activities as a function of human age is shown in Fig. 2. There is a positive correlation between increase in AFR reductase activity and PMRS. Comparing the relative activities of erythrocyte PMRS and AFR reductase, we observed an approximately 10-fold higher rate of AFR reductase compared to PMRS.

Discussion

ASC is a major aqueous-phase antioxidant and is a significant factor in the antioxidative capacity of plasma. ASC recycling serves to protect the erythrocyte against transmembrane oxidant stress.³ The ascorbate-driven reduction of the extracellular ascorbate free radical has been shown to be an electrogenic process, indicating that vectorial electron transport is involved in the reduction of extracellular ascorbate free radical.¹³ Vitamins C and E are directly linked by the role of vitamin C in regenerating vitamin E.^{14,15} Ascorbic acid has been proven to protect membrane and other hydrophobic compartments from oxidative damage by regenerating the antioxidant form of vitamin E.¹⁶ Ascorbate can recycle α -tocopherol in low-density lipoprotein (LDL) in the face of an oxidant stress and thus affords protection against oxidation.¹⁷ Thus, recycling of ASC also helps to protect or recycle α -tocopherol. Our observation of an increase in the

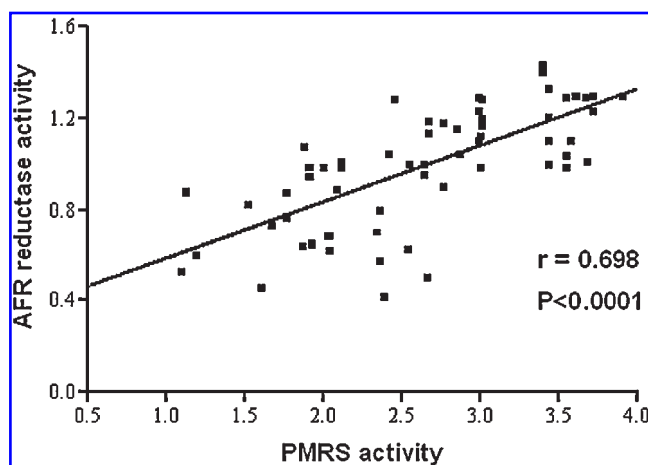


FIG. 2. Correlation between erythrocyte AFR reductase and PMRS activities. The PMRS activity was determined in the same blood samples for which AFR reductase activity is shown. AFR reductase activity is expressed in terms of μmol NADH oxidized/min per mL of packed red blood cells. PMRS activity is expressed as μmol ferrocyanide/mL PRBC per 30 min. The line at the top shows the range of age of human subjects taken for the study.

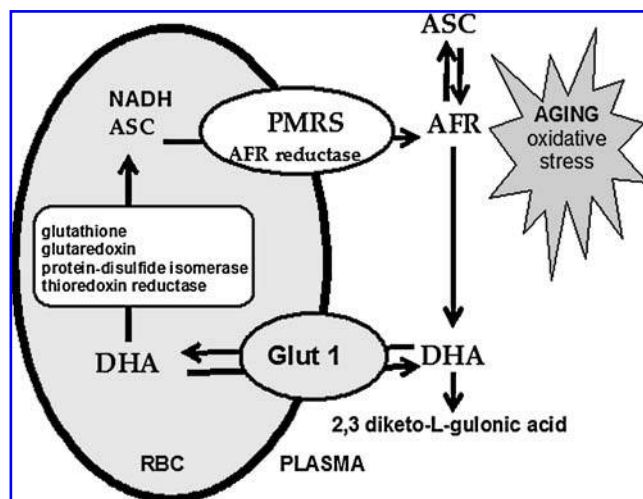


FIG. 3. Schematic representation of ascorbate recycling between erythrocytes and plasma. Under normal conditions, the PMRS and AFR reductase function to transfer reducing equivalents from intracellular electron donors to plasma. These electrons are used to reduce the AFR to reduced ASC. We propose that during aging the condition of oxidative stress is generated in the plasma, leading to higher rate of conversion of ASC to AFR. The increase in erythrocyte AFR reductase/PMRS activity is a compensatory mechanism to protect against increased oxidative stress.

activity of AFR reductase during human aging may thus provide a mechanism to maintain the antioxidant potential of the plasma during the increased oxidative stress that is encountered during aging.

Ascorbic acid recycling by erythrocytes has been reported to be increased by smoking.¹⁸ Smoking induces oxidative stress, and the increase in ASC recycling by erythrocytes has been explained as a secondary compensatory response in the human antioxidant defense.¹⁸ The plasma level of ASC in humans is known to be decreased during aging.^{19,20} The decrease in ASC has been found to be associated with an increase in plasma vitamin E and uric acid, an effect that has been explained as compensation in antioxidant defense of aging organism.¹⁹ A study of the British population shows that low blood vitamin C concentrations in the older individuals are strongly predictive of mortality.²¹ Rhesus monkeys exhibit some of the same characteristics of vitamin C metabolism as those in humans, and studies show that there is a significant decrease in plasma vitamin C level in aged monkeys.²² Significantly it has been reported that the activities of PMRS enzymes involved in transmembrane electron transport and recycling of ASC in the brain increase during aging and that this helps to protect the brain against age-related increases in oxidative and metabolic stress.²³ Our observation of an age-dependent increase in the activity of AFR reductase may also be explained to be due to a compensatory mechanism that becomes operative under condition of increased oxidative stress during aging in humans (Fig. 3).

Erythrocytes from aged individuals have been reported to have a reduced life span²⁴; however, no change has been observed in red cell aggregation, hematocrit, and whole blood viscosity during human aging.²⁵ In a recent study Penha-

Silva et al.²⁶ reported an increased erythrocyte resistance to hypotonic lysis in aged individuals; however, this study found no change in mean corpuscular volume (MCV) of erythrocytes as a function of human age. Despite the reduced life span of erythrocytes in older age and therefore a faster rate of turnover, it is difficult for this to significantly influence activity of AFR reductase.

Mechanisms to recycle ASC through transmembrane electron transport have been reported in chromaffin granules,²⁷ duodenal brush border,²⁸ and lysosomal membranes.²⁹ In all these cells, a transmembrane protein belonging to the family of cytochrome *b*₅₆₁ has been identified that is involved in ASC recycling. This protein has been isolated and the cDNA sequenced in a number of species, including humans.³⁰ Cytochrome *b*₅₆₁ has a unique structure, because it does not show homology to any known protein in genomic databases other than cytochrome *b*₅₆₁ from different species. In erythrocytes, the identification of the protein responsible for transmembrane electron transfer and ascorbate recycling has not yet been established. Van Duijn et al.³¹ carried out detailed studies based on reverse transcriptase-PCR analysis of reticulocytes but did not find the presence of cytochrome *b*₅₆₁ in erythrocytes. These results were also confirmed by western blot analysis with an anti-cytochrome *b*₅₆₁ serum. However, recently Su et al.³² confirmed that one of the cytochromes *b*₅₆₁ is present in human erythrocytes (the duodenal brush border cytochrome *b*) and contributes to the ability to reduce extracellular AFR; the same enzyme has ferricyanide reductase activity.

It is important to understand that the physiologic relevance of transmembrane ferrireductase activity in mature erythrocytes is not clear. The most likely function for this activity is in the developing erythroblast, where it is used to reduce ferric iron during its uptake and use in hemoglobin synthesis. The components of the hemoglobin-synthesizing system and the ability of iron uptake are lost during maturation of reticulocytes to erythrocytes.^{33,34} In contrast to transmembrane ferrireductase activity, the erythrocyte AFR reductase catalyzes the reduction of extracellular AFR to ASC, an important physiological process involved in ascorbate recycling. Interestingly, Su et al.³² have put forth the hypothesis that the same enzyme (Cyt *b*₅₆₁) has both AFR reductase and ferricyanide reductase activity in human erythrocytes, with one activity favored depending on the cellular milieu. Our observation of an approximately 10-fold higher erythrocyte AFR reductase activity compared to PMRS activity can be explained on the basis of higher physiological requirement of ascorbate recycling during the condition of increased oxidative stress as observed during aging.

On the basis of our results and in view of recent reports,^{10,23} we hypothesize that there are inherent compensatory mechanisms operating in the human body that provide protection against increased oxidative stress during aging. An alteration in the rate of these compensatory mechanisms may affect the rate of normal human aging.

Acknowledgment

This research was supported by the University Grants Commission, New Delhi, through a research grant (F 31-292/2005 SR) to S.I. Rizvi.

References

1. Bode AM, Cunningham I, Rose RC. Spontaneous decay of oxidized ascorbic acid (dehydro-L-ascorbic acid) evaluated by high-pressure liquid chromatography. *Clin Chem* 1990;36:807–1809.
2. Deutsch JC. Oxygen-accepting antioxidants which arise during ascorbate oxidation. *Anal Biochem* 1998;260:223–229.
3. Mendiratta S, Qu ZC, May JM. Erythrocyte ascorbate recycling: Antioxidant effects in blood. *Free Rad Biol Med* 1998;24:789–797.
4. Winkler BS. Unequivocal evidence in support of the nonenzymatic redox coupling between glutathione/glutathione disulfide and ascorbic acid/dehydroascorbic acid. *Biochim Biophys Acta* 1992;1117:287–290.
5. Wells WW, Xu DP, Yang YF, Rocque PA. Mammalian thioldisulfide transferase (glutaredoxin) and protein disulfide isomerase have dehydroascorbate reductase activity. *J Biol Chem* 1990;265:15361–15364.
6. May JM, Mendiratta S, Hill KE, Burk RF. Reduction of dehydroascorbate to ascorbate by the selenoenzyme thioredoxin reductase. *J Biol Chem* 1997;272:22607–22610.
7. Kennett EC, Kuchel PW. Plasma membrane oxidoreductases: Effects on erythrocyte metabolism and redox homeostasis. *Antiox Redox Signal* 2006;8:1241–1247.
8. VanDuijn MM, Tijssen K, VanSteveninck J, Van Den Broek PJ, Van Der Zee J. Erythrocytes reduce extracellular ascorbate free radicals using intracellular ascorbate as an electron donor. *J Biol Chem* 2000;275:27720–27725.
9. Rizvi SI, Maurya PK. Markers of oxidative stress in erythrocytes during aging in humans. *Ann NY Acad Sci* 2007;1100:373–382.
10. Rizvi SI, Jha R, Maurya PK. Erythrocyte plasma membrane redox system in human aging. *Rejuvenation Res* 2006;9:470–474.
11. May JM, Qu ZC, Cobb CE. Human erythrocyte recycling of ascorbic acid. *J Biol Chem* 2004;279:14975–14982.
12. Avron M, Shavit N. A sensitive and simple method for determination of ferrocyanide. *Anal Biochem* 1963;6:549–554.
13. Van Duijn MM, Van der Zee J, Van den Broek, PJ. The ascorbate-driven reduction of extracellular ascorbate free radical by the erythrocyte is an electrogenic process. *FEBS Lett* 2001;491:67–70.
14. Upston JM, Terentis AC, Stocker R. Tocopherol-mediated peroxidation of lipoproteins: implications for vitamin E as a potential antiatherogenic supplement. *FASEB J* 1999;13:977–994.
15. Packer JE, Slater TF, Wilson RL. Direct observation of a free radical interaction between vitamin E and vitamin C. *Nature* 1979;278:737–738.
16. Beyer RE. The role of ascorbate in antioxidant protection of biomembranes: interaction with vitamin E and coenzyme Q. *J Bioenerg Biomembr* 1994;26:349–58.
17. Jialal I, Vega GL, Grundy SM. Physiologic levels of ascorbate inhibit the oxidative modification of low density lipoprotein. *Atherosclerosis* 1990;82:185–191.
18. Lykkesfeldt J, Viscovich M, Poulsen HE. Ascorbic acid recycling in human erythrocytes is induced by smoking *in vivo*. *Free Rad Biol Med* 2003;35:1439–1447.
19. Siomek A, Gackowski D, Rozalski R, Dziaman T, Szpila A, Guz J, Olinski R. Higher leukocyte 8-oxo-7,8-dihydro-2-deoxyguanosine and lower plasma ascorbate in aging humans. *Antiox Redox Signal* 2007;9:143–150.
20. Simon JA. Vitamin C and cardiovascular disease: a review. *J Am Coll Nutr* 1992;11:107–125.
21. Fletcher AE, Breeze E, Shetty PS. Antioxidant vitamins and mortality in older persons: findings from the nutrition add-on study to the Medical Research Council Trial of the Assessment and Management of Older People in the Community. *Am J Clin Nutr* 2003;78:999–1010.
22. Preston AM, Bercovitch FB, Rodriguez CA, Lebron MR, Rivera CE. Plasma ascorbic acid concentrations in a population of rhesus monkeys (*Macaca mulatta*). *Contemp Top Lab Anim Sci* 2001;40:30–32.
23. Hyun DH, Emerson SS, Jo DG, Mattson MP, De Cabo R. Calorie restriction up-regulates the plasma membrane redox system in brain cells and suppresses oxidative stress during aging. *Proc Nat Acad Sci USA* 2006;103:19908–19912.
24. Kosower NS. Altered properties of erythrocytes in the aged. *Am J Hematol* 1993;42:241–247.
25. Feher G, Koltai K, Kesmarky G, Szapary L, Jurieskay I, Toth K. Hemorheological parameters and aging. *Clin Hemorheol Microcirc* 2006;35:89–98.
26. Penta-Silva N, Firmino CB, deFreitas Reis FG, Huss JC, deSouza TM, deFreitas MV, Netto Rde C. Influence of age on the stability of human erythrocyte membranes. *Mech. Ageing Dev* 2007;128:444–449.
27. Fleming PJ, Kent UM. Cytochrome b561, ascorbic acid, and transmembrane electron transfer. *Am J Clin Nutr* 1991;54:S1173–S1178.
28. McKie AT, Barrow D, Latunde-Dada GO et al. An iron-regulated ferric reductase associated with the absorption of dietary iron. *Science* 2001;291:1755–1759.
29. Zhang DL, Su D, Berczi A, Vargas A, Asard H. An ascorbate-reducible cytochrome b561 is localized in macrophage lysosomes. *Biochim Biophys Acta* 2006;1760:1903–1913.
30. Srivastava M. Genomic structure and expression of the human gene encoding cytochrome b561, an integral protein of the chromaffin granule membrane. *J Biol Chem* 1995;270:22714–22720.
31. Van Duijn MM, Buijs JT, Van der Zee J, Van den Broek PJE. The ascorbate:ascorbate free radical oxidoreductase from the erythrocyte membrane is not cytochrome_{b561}. *Protoplasma* 2001;217:94–100.
32. Su D, May JM, Koury MJ, Asard H. Human erythrocyte membranes contain a cytochrome b561 that may be involved in extracellular ascorbate recycling. *J Biol Chem* 2006;281:39852–39859.
33. Ponka P. Tissue-specific regulation of iron metabolism and heme synthesis: distinct control mechanisms in erythroid cells. *Blood* 1997;89:1–25.
34. Qian ZM, Morgan EH. Changes in the uptake of transferrin-free and transferrin-bound iron during reticulocyte maturation *in vivo* and *in vitro*. *Biochim Biophys Acta* 1992;1135:35–43.

Address reprint requests to:
 Dr. Syed Ibrahim Rizvi
 Department of Biochemistry
 University of Allahabad
 Allahabad 211002
 India

E-mail: sirizvi@gmail.com; rzv@rediffmail.com

Submitted: August 4, 2008
 Accepted: October 18, 2008