Nitric Oxide 23 (2010) 181–186

Contents lists available at ScienceDirect

Nitric Oxide

journal homepage: www.elsevier.com/locate/yniox

Chronic cigarette smoking alters erythrocyte membrane lipid composition and properties in male human volunteers

Pannuru Padmavathi [a], Vaddi Damodara Reddy [b], Godugu Kavitha [a], Maturu Paramahamsa [c], Nallanchakravarthula Varadacharyulu [a,*]

[a] Department of Biochemistry, Sri Krishnadevaraya University, Anantapur, AP 515055, India
[b] Department of Pediatrics, Pritzker School of Medicine, University of Chicago, Chicago, IL 60637, USA
[c] Department of Genetics, M.D. Anderson Cancer Center, Houston, TX 77030, USA

ARTICLE INFO

Article history:
Received 6 November 2009
Revised 10 May 2010
Available online 31 May 2010

Keywords:
Cigarette smoking
Erythrocyte membrane
Membrane fluidity
Na^+/K^+-ATPase
Nitric oxide

ABSTRACT

Cigarette smoking is a major lifestyle factor influencing the health of human beings. The present study investigates smoking induced alterations on the erythrocyte membrane lipid composition, fluidity and the role of nitric oxide. Thirty experimental and control subjects (age 35 ± 8) were selected for the study. Experimental subjects smoke 12 ± 2 cigarettes per day for 7–10 years. In smokers elevated nitrite/nitrate levels in plasma and red cell lysates were observed. Smokers showed increased hemolysis, erythrocyte membrane lipid peroxidation, protein carbonyls, C/P ratio (cholesterol and phospholipid ratio), anisotropic (γ) value with decreased Na^+/K^+-ATPase activity and sulfhydryl groups. Alterations in smokers erythrocyte membrane individual phospholipids were also evident from the study. Red cell lysate nitric oxide positively correlated with C/P ratio ($r = 0.565$) and fluorescent anisotropic (γ) value ($r = 0.386$) in smokers. Smoking induced generation of reactive oxygen/nitrogen species might have altered erythrocyte membrane physico-chemical properties.

Introduction

The prevalence of numerous health hazards (lung cancer, stroke, respiratory, cardiovascular diseases and osteoporosis) associated with cigarette smoking has been well documented [1]. Cigarette smoke is drawn through tobacco into active smoking mouth. The mainstream smoke consists of 8% tar (nicotine, carcinogens, etc.) and 92% gaseous constituent components (carbon monoxide, ammonia, hydrogen cyanide, etc.) which enter blood [2]. As a result, blood constituents, plasma, RBC, platelets and WBC are exposed to smoke contents. High doses of reactive oxygen species (ROS) present in cigarette smoke leads to inability of the biological system to cope with their production and result in chemical modification of biological molecules, metabolic malfunctions and damage to biological macromolecules [3].

Erythrocyte membranes have been used in several physiological, biochemical and toxicological investigations, and were demonstrated to be an ideal membrane for membrane investigations [4]. Maintenance of the appropriate membrane lipid composition and fluidity are critical for the proper functioning of integral membrane proteins, membrane bound enzymes, receptors and ion channels [5,6]. Many studies have shown that disorders of circulatory system are linked to insufficient rheological behavior of erythrocytes [7]. It has also been proposed that cell membrane abnormalities of erythrocytes are an etiological factor in hypertension, including functional abnormalities such as transmembrane cation fluxes and structural changes of the cell membranes [8]. Surprisingly, investigations reveal that 23 identified biomarkers are lipids affecting structure and function of membrane [9]. Nitric oxide (NO), the end product of the enzyme nitric oxide synthase, influences various physiological processes in every organ and tissue. It has a remarkably broad spectrum of functions such as regulation of vascular tone, neurotransmission, antimicrobial defense mechanisms and immunomodulation [10,11]. NO participates in the regulation of the rheological behavior of erythrocytes and determines RBC mechanical behavior and at some critical concentration, it appears necessary for preserving the cellular mechanical property and is also effective in modulating membrane fluidity [12,13]. Particular attention has been given to NO in cigarette smoke-induced deleterious effects. However, influence of NO on red cell membrane fluidity is not clear in cigarette smokers.

Since the functions of proteins embedded in membranes are modulated by the composition and properties of the lipid bilayer environment and the lipid bilayer influences both the accessibility of protein sites and NO interactions. The present study is aimed to assess erythrocyte membrane oxidative damage, composition and fluidity, hemolytic behavior, and the role of nitric oxide in the

* Corresponding author. Fax: +91 8554 255244.
E-mail address: vdp_1975@yahoo.co.in (N. Varadacharyulu).

1089-8603/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.niox.2010.05.287

causation of cigarette smoke-induced effects on red cells of cigarette smokers.

Materials and methods

Chemicals

All standards (phosphatidylserine, phosphatidylcholine, phosphatidylethanolamine, cholesterol sphingomyelin and malondialdehyde) and the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Silica gel G60 sheets for TLC were purchased from Merck (Darmstadt, Germany). All other chemicals were obtained from Qualigens (Glaxo India Ltd., Mumbai, India) and Sisco Research Laboratories (SRL, Mumbai, India).

Subjects

Thirty human male volunteers in each group, aged 35 ± 8 residing in Anantapur, India taking local diet and smoking for 7–10 years at least 12 ± 2 cigarettes per day were chosen as experimental subjects. All the subjects were using cigarettes without a filter. Controls (age, sex and diet matched) who did not smoke were selected for the study. All subjects were free from chronic diseases, illness, use of any tranquillizers, drugs and anesthetics. All the volunteers were well informed about the experimentation and their written consent was obtained. Institutional ethical committee approved this study. Samples were obtained after the subjects had fasted over night and smoked their last cigarette 8 h before the experimental procedure began. Venous blood was drawn into heparin-treated evacuated tubes and centrifuged immediately at 1500*g* for 10 min at 4 °C to separate plasma and red cells.

Measurement of nitrite/nitrates levels

Erythrocyte lysate was prepared [14], plasma and red cell lysate samples were treated with 30% zinc sulfate to deproteinize samples followed by centrifugation at 4000*g* for 5 min. Nitrite was determined from 1.0 ml aliquots of plasma and erythrocyte lysate using Griess reagent (1% sulfanilamide, 2.5% phosphoric acid and 0.1% 1-naphthylethylene diamine). One milliliter aliquots of the supernatant were swirled for 90 min separately with activated cadmium granules for the conversion of nitrite to nitrate and then Griess reagent was added. Nitrite concentrations were estimated using a standard curve developed with sodium nitrite [15].

Osmotic fragility of red blood cells

Isolated red blood cells were incubated in different concentrations of NaCl ranging from 0.1% to 0.9% for 30 min with gentle stirring, hemoglobin released into supernatant from the red cells was determined after a spin at 2500*g* for 10 min absorbance was measured at 540 nm [16].

Erythrocyte membrane studies

Erythrocyte membrane was prepared [17] and the classical thiobarbiturate assay was used for the estimation of erythrocyte membrane MDA levels [18]. The concentration of protein carbonyls was determined using 2,4-dinitrophenylhydrazine (DNPH) assay [19,20]. Erythrocyte membrane total sulfhydryl groups were determined [21]. The activity of Na^+/K^+-ATPase was measured by estimating the phosphorus liberated after the incubation of erythrocyte membrane in a reaction mixture containing the substrate ATP with the co-substrate elements at 37 °C for 15 min. The reaction was arrested by adding 1.0 ml of 10% TCA. The phosphorus content from the TCA supernatants was then determined [22]. Erythrocyte membrane protein concentration was estimated [23].

Erythrocyte membrane lipid extraction and analysis

Erythrocyte membrane lipids were extracted [24]. To the lysed membrane preparations, 5 ml of methanol was added followed by chloroform. After 30 min, the same was filtered to collect filtrate and the residue was again subjected to same step and filtered again. The filtrates were pooled up and used for lipid analysis. Lipids were extracted from a portion of the erythrocyte membrane suspension with iso-propanol and chloroform and aliquots were taken for estimation of cholesterol [25] and phospholipids [26], respectively.

Erythrocyte membrane individual phospholipids were separated on silica gel H (Merck) using two dimensional thin layer chromatography with chloroform–methanol–aqueous ammonia 65:35:5 (v/v) as the first solvent and chloroform–acetone–methanol–acetic acid–water 50:20:10:10:5 (v/v) as the second solvent. The fractions were located with iodine vapors and scraped from the plate and the phospholipids were measured as inorganic phosphorus after digestion with perchloric acid [27].

Measurement of membrane fluidity

The quantitative measurement of membrane fluidity was performed by the fluorescence polarization technique with DPH as fluorescence probe [28]. Membrane preparations (50 μg protein) were suspended in 50 mmol/L Tris–HCl buffer (pH 7.4), mixed with DPH prepared from a stock solution of 5 mmol/L DPH solubilized in tetrahydrofurans, and incubated at 37 °C for 30 min. Fluorescence polarization was determined using a Hitachi fluorescence spectrophotometer (Hitachi, Tokyo, Japan) equipped with rotating polarizing filters with samples held at 25 °C. Samples were excited at 360 nm and the emission intensity was read at 435 nm. Polarization (P) and fluorescence anisotropy (c) were calculated using the equation. $P = I_{VV} - I_{VH \cdot G}/I_{VV} + I_{VH \cdot G}$. Where I_{VV} and I_{VH} are the intensities measured parallel and perpendicular to the vertical axis of the excitation beam, and G is the correction factor $I_{VH}/I_{HH} \cdot \gamma$ is calculated using the formula $\gamma = 2P/(3 - P)$.

Statistical analysis

Data were subjected to statistical analyses, values are means of ±SD of 30 subjects in each group. The data were normally distributed and student t-test was performed for finding significant difference between the groups. A $p < 0.05$ was considered statistically significant. Correlations between variables were assessed with Pearson's correlation coefficient (r).

Results

Base line characteristics of controls and cigarette smokers were presented in Table 1. Nitrite and nitrate levels are considered to be reliable indicators of nitric oxide generation. Increased concentrations of nitrite/nitrate levels in plasma and erythrocyte lysate of cigarette smokers suggested overproduction of nitric oxide when compared to controls (Fig. 1). Fig. 2 shows the extent of lipid peroxidation in erythrocyte membrane of control and cigarette smokers. Erythrocyte membrane lipid peroxidation was significantly ($p < 0.05$) higher in cigarette smokers than in controls. Erythrocyte membrane carbonyl groups and sulfhydryl groups are furnished in