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Alterations in oxidative stress biomarkers associated with mild hyperlipidemia and smoking

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ABSTRACT

Oxidative stress may increase the risk of atherosclerosis. The association of mild forms of hyperlipidemia, particularly primary hypertriglyceridemia, with oxidative stress has not been fully investigated. The aim of this study was to assess the alterations in oxidative stress biomarkers associated with three major types of mild untreated hyperlipidemia (hypercholesterolemia, hypertriglyceridemia and combined hyperlipidemia) in nonsmoker and smoker individuals. Five biomarkers were measured in 139 adult healthy men (83 nonsmokers and 56 smokers, ages 18–75), which included normolipidemic and hyperlipidemic subjects. Triglyceride levels were associated with a significant main effect on ferric reducing antioxidant power (FRAP) and 8-*iso*-prostaglandin $F_{2\alpha}$ ($iPF_{2\alpha}$) levels in plasma ($p < 0.05$ and $p < 0.005$, respectively). Smokers with hypercholesterolemia, hypertriglyceridemia and combined hyperlipidemia had alterations in 1, 3 and 2 oxidative stress biomarkers compared to nonsmoker normolipidemics. Smokers (including normolipidemics and hyperlipidemics) had higher plasma FRAP (120.8 *vs.* 102.0 μM quercetin/l, $p < 0.05$) and erythrocyte catalase activity (5125 *vs.* 4093 U/g Hb, $p < 0.01$), while they had lower erythrocyte glutathione peroxidase activity (20.3 *vs.* 23.0 U/g Hb, $p < 0.05$) compared to nonsmokers. These findings show that mild forms of hyperlipidemia, particularly in smokers, are associated with alterations in some oxidative stress biomarkers.

1. Introduction

Reactive oxygen species (ROS), including free radicals, are involved in normal biochemical processes (Bedard and Krause, 2007), but their overproduction can cause damage to various macromolecules (Halliwell and Gutteridge, 2007). Oxidative stress is caused by an imbalance between the production of ROS and the ability of the human protective physiological processes to detoxify these species.

Oxidative stress can cause or aggravate several human diseases (Firuzi et al., 2008; Nunomura et al., 2001); its role is especially important in atherosclerosis and coronary heart diseases (Harrison et al., 2003). Oxidative stress independent of other risk factors, has been reported to be associated with carotid atherosclerosis (Kotani et al., 2010) and it probably should be considered as a therapeutic target and kept under control in order to manage the risk of atherosclerotic cardiovascular diseases (Munzel et al., 2010).

Hyperlipidemia is associated with constant increases in cholesterol and/or triglyceride blood levels and is a major risk factor for development of cardiovascular diseases (Anderson et al., 1987). Hypercholesterolemia (HC) induces the production of ROS such as superoxide anion, through enzymes like NADPH oxidase and xanthine oxidase as well as other mitochondrial ROS sources (Munzel et al., 2010). Several investigators have reported that biomarkers of oxidative stress are increased in familial HC (Munzel et al., 2010; Pirincciogluet al., 2010; Real et al., 2010; Reilly et al., 1998) and familial combined hyperlipidemia (simultaneous increase of cholesterol and triglyceride levels) (Martinez-Hervas et al., 2008). However, there are only few reports on the role of oxidative stress in hypertriglyceridemia (HTG) (de Man et al., 2000; Pronai et al., 1991) most of these reports being focused on oxidative stress in postprandial HTG (Cardona et al., 2008), but not primary HTG. On the other hand, investigators have usually studied moderate to severe increases of serum lipid levels such as in

Abbreviations: FRAP, ferric reducing antioxidant power; CAT, catalase; CHL, combined hyperlipidemia; GPX, glutathione peroxidase; HC, hypercholesterolemia; HTG, hypertriglyceridemia; $iPF_{2\alpha}$, 8-*iso*-prostaglandin $F_{2\alpha}$; SOD, superoxide dismutase.

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familial hyperlipidemia, which is associated with highly elevated lipid levels from early age, but asymptomatic mild increases of lipid levels have not been studied in detail.

Cigarette smoking is another important risk factor for development of atherosclerotic cardiovascular diseases (Bartecchi et al., 1994). Cigarette smoke contains many oxidants (Halliwell and Gutteridge, 2007; Mur et al., 2004) and it can be associated with oxidative stress in smokers (Pignatelli et al., 2001; Yamaguchi et al., 2005).

Although, hyperlipidemia and smoking are both associated with oxidative stress, there are only few reports on the influence of concurrent presence of these factors on oxidative stress biomarkers (Burke and Fitzgerald, 2003; Heitzer et al., 1996). It has been shown that smoking and HC synergistically cause endothelial dysfunction in forearm resistance vessels (Heitzer et al., 1996). However, to our knowledge, the association of concurrent presence of smoking and different types of hyperlipidemia with various oxidative stress biomarkers has not been studied before. Since oxidative stress associated with hyperlipidemia and smoking is an important mechanism for increased risk of atherosclerosis in these conditions, study of the interaction of these risk factors and the way their simultaneous presence can change oxidative stress biomarkers is of crucial importance.

In this study, we assessed the association of smoking and mild untreated hyperlipidemia with oxidative stress biomarkers in plasma and erythrocytes of clinically healthy men. Diverse biomarkers were chosen to study oxidative stress from different aspects. We measured ferric reducing antioxidant power (FRAP) to evaluate the total antioxidant capacity and total 8-*iso*-prostaglandin $F_{2\alpha}$ ($iPF_{2\alpha}$), an important indicator of lipid peroxidation, in plasma. We also assessed three antioxidant enzymes comprising catalase (CAT), glutathione peroxidase (GPX) and superoxide dismutase (SOD) in erythrocytes.

2. Materials and methods

2.1. Subjects

One hundred thirty-nine adult healthy men were recruited from individuals who were referred to a clinical laboratory in the city of Shiraz, Iran for a routine check-up analysis. Individuals were thoroughly informed about the research project and written informed consents were obtained from all participants. The study was approved by the Ethics Review Board for human studies of the Shiraz University of Medical Sciences and was in accordance with the guidelines of the declaration of Helsinki.

Men at the age of 18 or older and with a body mass index (BMI) of lower than 30, were included in the study. A detailed medical history was taken and individuals with a past or present history of any serious illness such as cardiovascular (including hypertension), hepatic, renal, inflammatory, endocrine or neurological disorders were excluded from the study. Individuals who were taking lipid lowering therapy or any other medications (except for prophylactic aspirin) or antioxidant agents such as vitamins, as well as the individuals who reported any illness in the past 2 weeks such as common cold and flu were excluded from the study. Subjects with LDL-cholesterol (LDL-c) and triglyceride levels higher than 190 mg/dl and 450 mg/dl, respectively, were not included, because the focus of the study was on mild hyperlipidemia. Nonsmoker individuals had never smoked or had quit at least 5 years before sample collection.

The participants included 83 nonsmokers and 56 smokers. Each group was subdivided in normolipidemia (LDL-c < 130 mg/dl, triglyceride < 150 mg/dl), hypercholesterolemia (HC, LDL-c > 130 mg/dl, triglyceride < 150 mg/dl), hypertriglyceridemia (HTG, LDL-c < 130 mg/dl, triglyceride > 150 mg/dl) and combined hyperlipidemia groups (CHL, LDL-c > 130 mg/dl, triglyceride > 150 mg/dl) according to the National Cholesterol Education Program (NCEP) (2001). Nonsmoker subjects with normolipidemia, HC, HTG and CHL were designated as subgroups 1, 2, 3 and 4, respectively. Smokers with normolipidemia, HC, HTG and CHL were nominated as subgroups 5, 6, 7 and 8, respectively.

According to the National Cholesterol Education Program (NCEP) (2001), LDL-cholesterol (LDL-c) and cholesterol levels of 130–160 and 200–240 mg/dl, respectively, are defined as "borderline high". The mean levels of LDL-c and cholesterol in our hyperlipidemic groups lied in these ranges. Furthermore, none of the individuals had a LDL-c level of higher than 190, which is considered "very high". Triglyceride mean levels were also 251.5 mg/dl or lower, which are in the low range of "high triglyceride" definition (200–499 mg/dl). None of the subjects had a "very high" triglyceride level (>500 mg/dl) (NCEP, 2001).

2.2. Blood sample collection

Blood samples were taken after overnight fasting in two potassium EDTA containing vacutainers. One of the tubes was centrifuged at 1500*g* for 10 min and plasma was separated for measurement of ferric reducing antioxidant power (FRAP) and 8-*iso*-prostaglandin $F_{2\alpha}$ ($iPF_{2\alpha}$) and kept at −80 °C till the time of analysis. FRAP measurements were performed within one week after sample collection, because long storage may cause alterations of FRAP values (Firuzi et al., 2006b). For measurements of $iPF_{2\alpha}$, 1 μl of butylhydroxytoluene (BHT) 10 mM was added to each 100 μl of plasma before transferring the aliquots to −80 °C. The second blood tube was centrifuged at 3000*g* for 10 min, plasma was discarded and red blood cells (RBCs) pellet was washed three times with ice cold physiological saline (NaCl 0.9%) to ensure the removal of plasma, leukocytes and platelets. RBCs were hemolysed in sterile ice cold double distilled water in a 1:5, RBC:water ratio. Lysed RBC samples were immediately transferred to −80 °C. Samples were kept on ice throughout the above mentioned procedures.

2.3. Reagents

Ammonium sulfate, Brij 35, butylhydroxytoluene (BHT), chloroform, dihydronicotinamide adenine dinucleotide phosphate (NADPH), ethanol, hydrochloric acid (HCl), hydrogen peroxide (H_2O_2), methanol, potassium dihydrogen phosphate (KH_2PO_4), potassium hydroxide (KOH), sodium hydrogen phosphate (Na_2HPO_4), sodium dihydrogen phosphate (NaH_2PO_4), sodium carbonate (Na_2CO_3), sodium acetate (CH_3COONa), tert-butyl hydroperoxide (TBH), 2,4,6-Tris(2-pyridyl)-*s*-triazine (TPTZ) were obtained from Merck Chemicals (Darmstadt, Germany, http://www.merck.de). Albumin from bovine serum, cupric chloride ($CuCl_2$), Drabkin's reagent, ethylenediaminetetraacetic acid (EDTA), ferric chloride ($FeCl_3$), glutathione (GSH), glutathione reductase (GSSG-R), nitroblue tetrazolium (NBT), sodium azide (NaN_3), Tris base, Tris–HCl, xanthine and xanthine oxidaze were purchased from Sigma–Aldrich (St. Louis, MO, http://www.sigma-aldrich.com). Quercetin was obtained from Acros Organics (Geel, http://www.acros.com). Spectrophotometric measurements were carried out by a Bio-Tek spectrophotometer (Uvikon XL) and a Bio-Rad microplate reader (680).

2.4. Ferric reducing antioxidant power (FRAP) assay

FRAP assay was performed according to the method of Benzie and Strain (1996) with minor modifications. FRAP solution was prepared by mixing 10 ml of acetate buffer 300 mM pH 3.6, 1 ml of ferric chloride hexahydrate 20 mM in distilled water and 1 ml of 2,4,6-*tris*(2-pyridyl)-*s*-triazine 10 mM in HCl 40 mM. Ten microliters of plasma was mixed with 190 μl of FRAP solution in 96-well microplates in duplicate. One blank sample was also used for each plasma sample, which contained the same amount of plasma and 190 μl of acetate buffer. The absorbance was measured at 595 nm after 30 min of incubation at room temperature by a microplate reader. FRAP value of plasma samples were calculated in reference to quercetin, an antioxidant flavonoid, which was tested at a final concentration of 5 μM.

2.5. Total 8-iso-prostaglandin $F_{2\alpha}$

Plasma $iPF_{2\alpha}$ exists as free form as well as esterified to lipoproteins. In order to measure total $iPF_{2\alpha}$ a hydrolysis step was undertaken with KOH 15% at 40 °C for 1 h to release esterified $iPF_{2\alpha}$. Afterwards, plasma proteins were precipitated by a solution of 30% ethanol containing 0.01% butylhydroxytoluene (BHT) and the pH was adjusted to 7 by a solution of KH_2PO_4 1.25 M. Total $iPF_{2\alpha}$ levels were then determined by a commercially available kit (Cayman Chemical Co.) according to the instructions of the manufacturer.

2.6. Catalase activity in erythrocytes

Erythrocyte catalase (CAT) activity was spectrophotometrically measured according to the method of Aebi (1984). The assay is based on the determination of hydrogen peroxide decomposition in the presence of CAT present in erythrocytes, by measuring absorbance change at 240 nm. The final reaction mixture (3.0 ml) contained 2.8 ml of sodium phosphate buffer 50 mM pH 7.2, 100 μl of hemolysate diluted with deionized water (1:3) and 100 μl of hydrogen peroxide 30 mM. The absorbance was determined by a spectrophotometer after 1 min. Proper blank samples were run in parallel. Each unit of CAT activity is defined as the amount of the enzyme required to decompose 1 μmol of hydrogen peroxide ($\varepsilon = 43.6$). Hemoglobin levels in RBC lysates were measured by Drabkin's reagent.