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Basic Research

Enhanced oxidative stress in neutrophils from hyperlipidemic guinea pig

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Abstract

Inhibitors of 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase are antilipidemic agents (statins) widely used for the prevention of cardiovascular diseases. Recent studies have suggested that the overall benefits of statin therapy cannot be accounted for solely by its antilipidemic effect. To obtain further insight into the mechanism of action of statins, we studied the effect of pitavastatin on the generation of reactive oxygen species (ROS) by peritoneal polymorphonuclear leukocytes (PMN) obtained from control and hyperlipidemic guinea pigs. Flow cytometric analysis revealed that the amount of ROS generated by PMN from the hyperlipidemic animals that had been administered a laurate-containing diet (LD) for 4 weeks was larger than that from the normal diet (ND) group (837% increase, ND; 82.17 arbitrary units, LD; 688.10 arbitrary units, $P < 0.01$, $n = 6$). Administration of pitavastatin to the LD group significantly decreased plasma levels of total cholesterol (TC) and low-density lipoprotein (LDL) with a reduction in ROS generation by PMN (19% decrease, LD control; 688.10 arbitrary units, LD+pitavastatin; 556.87 arbitrary units, $P < 0.01$, $n = 6$). Western blotting analysis revealed that the expression of protein kinase C α (PKC α) and βI was higher in PMN from the LD group than in PMN from the ND group (PKC α; 74% increase, PKC βI; 339% increase, $P < 0.05$, $n = 4$, respectively). Furthermore, expression of NADPH oxidase gp91phox in PMN from the LD group was higher than that in PMN from the ND group (18% increase, $P < 0.05$, $n = 4$). By administration of pitavastatin to the LD group, the expression of PKC α, βI and gp91phox was suppressed compared with the control LD group (PKC α; 41% decrease, PKC β; 28% decrease, gp91phox; 56% decrease, $P < 0.05$, $n = 4$, respectively). These results indicate that PMN from hyperlipidemic animals is associated with an accelerated respiratory burst of ROS by increasing the expression of PKC α, βI and gp91phox, and pitavastatin inhibits this by suppressing the expression of those proteins.

Keywords: Hyperlipoproteinemia; HMG-CoA reductase inhibitor; Fluorescence; Leukocytes; Oxidative stress

1. Introduction

Recent studies have suggested that inhibitors of 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase (statins) have greater beneficial effects on cardiovascular functions than what would be expected simply from the decrease in plasma cholesterol levels they induce [1–5]. Increasing evidence suggests that reactive oxygen species (ROS) oxidize low-density lipoprotein (LDL), and contribute to the formation of foam cells in arterial walls during the early stages of atherosclerosis, and that the oxidized LDL further enhances the generation of ROS by polymorphonuclear leukocytes (PMN) [6–9]. However, the factors responsible for oxidative modification of LDL are yet to be fully clarified. Although close correlations between PMN-derived ROS and cardiovascular risk factors, such as hypertension and hyperglycemia, have been suggested [10], the relationship between hyperlipidemia and PMN-derived ROS and the effect of statins on these two factors remain to be elucidated. Pitavastatin, (+)-monocalcium bis{(3R,5S,6E)-7-[2-cyclopropyl-4-(4-flourophenyl)-3-quinolyl]-3,5-dihydroxy-6-heptenoate}, is a long-acting potent statin [11,12] which strongly reduces plasma levels of total cholesterol (TC) and triglycerides (TG) [13]. NADPH oxidase is the primary source for ROS generated by PMN [14–17], and protein kinase C (PKC) plays a critical role in activation of the enzyme.

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We previously showed that statins reduced both oxidative stress and smooth muscle cell migration via PKC- and phospolipase D-dependent mechanisms [18]. The present study describes the enhanced oxidative stress associated with hyperlipidemia through the possible involvement of PKC and NADPH oxidase and the effect of pitavastatin on ROS generation by PMN.

2. Materials

Catalase, Cu/Zn-superoxide dismutase (SOD), diphenyleneiodonium (DPI) and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma Chemical Co. (St. Louis, MO). Staurosporine and 2′, 7′-carboxy dichlorodihydrofluorescein diacetate bis-acetoxymethyl ester cytometric method with a fluorescent probe (CDCFH) were purchased from Wako Pure Chemical Co. (Osaka, Japan) and Molecular Probe Co. (Eugene, Oregon), respectively. Pitavastatin was the generous gift of Kowa Co. Ltd. (Tokyo, Japan). Palm oil was purchased from Fujiseiyu Co. Ltd. (Osaka Japan). Main fatty acids in the palm oil were laurate (48%) and myristate (16%).

3. Methods

3.1. Animals and diets

Male Hartley guinea pigs (5 weeks of age) were purchased from SLC Japan (Shizuoka, Japan). A standard laboratory chow (ND) (RC-4: Oriental Yeast Co. Ltd., Tokyo, Japan) was given to the control group and feed containing 15% palm oil (LD) was given to the hyperlipidemic group. The amount of cholesterol in the laurate diet was adjusted to 0.04%.

Guinea pigs were divided into four groups with similar levels of plasma TC, TG and LDL-C. Two groups were fed either ND or LD for 4 weeks. Another two groups were fed the same diets for 4 weeks and given water containing pitavastatin water (1 mg/kg) for the last 2 weeks.

All studies were approved by the Institutional Animal Care and Use Committee of Osaka City University Medical School.

3.2. Analysis of lipids

Blood samples were collected from four animal groups in full stomach and plasma levels of TC and TG were measured by the enzymatic method [19]. LDL was determined as previously reported [20].

3.3. Preparation of PMN

PMN were obtained from the peritoneal fluid 16 h after intraperitoneal injection of 100 mL/kg of 2% casein containing 5 mmol/L Tris–HCl buffer (pH 7.4) and 0.15 mol/L NaCl. Under light ether anesthesia, 50 mL of Ca^{2+}-free phosphate-buffered saline (PBS, pH 7.4) was injected intraperitoneally. After 16 h, peritoneal lavage fluid was collected in plastic tubes, and filtered through a nylon mesh (12 μm) to eliminate cell debris. After removing erythrocytes by hypotonic lysis at 4 °C, PMN were centrifuged and the precipitate was resuspended in PBS. After calculating cell number by light microscopy, cells were suspended in PBS (1×10^6 cells/mL) and immediately used for experiments. The purity of PMN in the samples was higher than 95%.

3.4. Flow cytometric analysis

To carefully distinguish PMN from other types of leukocytes, we utilized a flow cytometric method with a fluorescent probe that is sensitive to hydroperoxide and is trapped within viable cells in a nonfluorescent form and is then converted to fluorescent dichlorofluorescein by hydroperoxides. Generation of ROS by PMN was analyzed by the gated-flow cytometry method with a FACS Calibur (Becton Dickinson) as described previously [21,22]. After incubation of PMN in 500 μL PBS containing 5 μmol/L CDCFH at 37 °C for 15 min, fluorescence intensity was determined.

3.5. Western blotting analysis

PMN were incubated in 1 mL of 0.9% NaCl containing 20% trichloroacetic acid at 4 °C for 15 min, and then centrifuged at $10{,}000 \times g$ for 5 min. The precipitate was dissolved by sonication for 10 s in 80 μL of 9 M urea containing 2% TritonX-100 and 1% dithiothreitol. Total protein was assayed by BCA analysis (Pierce). The mixtures were dissolved with 20 μL of 10% lithium dodecylsulfate containing 0.01% bromophenol blue (pH 7.4). Total protein (15 μg) was subjected to SDS-PAGE (10% gel). The electrophoresed proteins were transferred to a nylon membrane, and analyzed by Western blotting using antibodies specific to PKC α, PKC βI, PKC βII and p67phox (Santa Cruz Biotechnology). The antibodies specific to C-terminal CSESTKRKLASAV of p47phox and C-terminal KQSISNSESGPRG of gp91phox were produced in rabbits. Immunoreactive protein bands were visualized by the enhanced chemiluminescence method.

3.6. Statistical analysis

Statistical analysis was performed by analysis of variance and Student's *t*-test. All values are means ± S.D. derived from six animals. Values of $P < 0.05$ were considered statistically significant.

4. Experimental results

4.1. Effect of pitavastatin on lipid levels in plasma

To confirm the hypolipidemic effect of pitavastatin, we measured plasma levels of TC, TG and LDL (Fig. 1). Plasma levels of TC and LDL were significantly higher in the LD