Mechanisms Underlying Diquertin-Mediated Regulation of Neutrophil Function in Patients with Non-Insulin-Dependent Diabetes Mellitus

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We studied the effects of dihydroquercetin (3.3.4.5.7-pentahydroxyflavanone, a new Russian patented preparation) on functional activity of polymorphonuclear neutrophils from patients with non-insulin-dependent diabetes mellitus. Flavonoids (quercetin and its derivative dihydroquercetin) dose-dependently suppressed generation of anion radicals and hypochlorous acid and production of malonic dialdehyde during oxidation of neutrophil membranes. Dihydroquercetin decreased activities of protein kinase C and myeloperoxidase in activated polymorphonuclear neutrophils and could bind transition metals (Fe^{2+}). These properties determine the ability of dihydroquercetin to decrease *in vitro* functional activity of polymorphonuclear neutrophils from patients with non-insulin-dependent diabetes mellitus.

Key Words: *neutrophils; non-insulin-dependent diabetes mellitus; dihydroquercetin; superoxide anion radical; lipid peroxidation*

Interest in oxidative stress, which serves as a key pathogenetic mechanism for the development and progression of various diseases (*e.g.*, complications of non-insulin-dependent diabetes mellitus, NIDDM), markedly increased in recent years. Particular attention was given to polymorphonuclear neutrophils (PMN). These cells can generate superoxide anion radical ($O^{\bullet}_2$) and other reactive oxygen species (ROS) in the NADPH oxidase and myeloperoxidase system and during oxidation of arachidonic acid. Superoxide dismutase (SOD) catalyzes reduction of $O^{\bullet}_2$ to H_2O_2, which undergoes degradation to hydroxyl radicals. Hypochlorous acid (HOCl) is a potent endogenous oxidant produced during activation of PMN with myeloperoxidase. H_2O_2, $O^{\bullet}_2$, and HOCl possess antimicrobial properties, but induce damage to vascular endothelium and contribute to the development of insulin resistance (pathogenetic mechanism of NIDDM).

Previous studies showed that lipid peroxides possess angiotropic activity. Antioxidants are used for the therapy of patients with diabetes mellitus. Dihydroquercetin is a new Russian patented preparation from larch wood. This substance is a chemical analogue of quercetin hydrated by the heterocyclic fragment. Dihydroquercetin possesses antioxidant properties [2, 12], is more available than other biological flavonoids, and possesses antiplatelet and capillary-protective properties comparable to those of other flavonoids [3]. Therefore, dihydroquercetin holds much promise for the therapy of patients with diabetes mellitus.

Here we studied the effects of dihydroquercetin on functional activity of PMN from NIDDM patients.

MATERIALS AND METHODS

Twelve men with NIDDM and 8 healthy donors (43-70 years) were examined.

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The mean history of NIDDM was 10-29 years. According to criteria of the European Diabetes Police Group (1993), the patients were in a satisfactory state and had compensated carbohydrate metabolism. The content of glycosylated hemoglobin in the blood (less than 7.5%) was measured by the latex inhibition of immunoagglutination using Hemoglobin Alc Reagent kits (Boeringer Mannheim).

The suspension of PMN was obtained from the whole blood stabilized with 3.8% sodium citrate (ratio 9:1). Gradient centrifugation on Percoll (Pharmacia) was followed by 2-fold washout and suspending in a buffer containing 120 mM NaCl, 4 mM KCl, 2 mM glucose, and 25 mM Tris (pH 7.4). PMN count in the suspension was 11.1×10^6 cells/ml. Neutrophil viability evaluated by trypan blue exclusion was 95%.

For evaluation of antioxidant activity of flavonoids, dihydroquercetin (Vilar) and quercetin (Sigma) were added to PMN suspension to final concentrations of 1, 10, and 100 μM and incubated at 37°C for 5 min with constant stirring.

PMN were stimulated with phorbol myristate acetate (PMA, Sigma) in a final concentration of 1 μg/ml. PMN were incubated with PMA at 37°C for 15 min. The reaction was stopped by placing on ice. The suspension was centrifuged at 800*g* for 10 min.

Generation of $O^{\bullet}$ by PMN was determined by reduction of ferricytochrome C inhibited by SOD. Cytochrome C (0.6 mg) was added to the suspension (0.5 ml) containing 10^6 PMN. The mixture was incubated at 37°C for 20 min. Absorption of cytochrome C was measured after introduction of SOD (310 U) to the supernatant at 550 nm. The amount of generated $O^{\bullet}$ was estimated by the concentration of reduced cytochrome C (absorption coefficient 21.0 $mM^{-1}cm^{-1}$).

HOCl was detected by chlorination of taurine [13]. The suspension of PMN (10^6) activated with PMA was incubated with 15 mM taurine in 154 mM NaCl (pH 7.4) at 37°C for 60 min. Potassium iodide (20 mM) was added to the supernatant. Absorption was measured at 350 nm. The absorption coefficient was 22.9 $mM^{-1}cm^{-1}$.

Lipid peroxidation (LPO) was induced by incubation of PMN with 0.01 mM $FeSO_4$ and 0.05 mM (L+)-ascorbic acid at 37°C for 15 min. Then the suspension of PMN was rapidly frozen. The intensity of LPO was determined by accumulation of 2-thiobarbituric acid-reactive products. An equivalent amount of malonic dialdehyde (MDA) was measured spectrofluorometrically with some modifications [14]. MDA content was expressed in relative units of fluorescence per 10^6 PMN.

Statistical treatment was performed by the method of paired and independent samples. The results were analyzed by Student's *t* test. The data are expressed as $M\pm m$.

RESULTS

Nonactivated PMN from NIDDM patients and healthy donors did not generate $O^{\bullet}$ and HOCl. It was probably related to compensation of carbohydrate metabolism in NIDDM patients. $O^{\bullet}$ generation in healthy donors and, particularly, in patients was intensified after activation of neutrophils with PMA. The amount of HOCl produced by PMN did not differ in patients and healthy donors. Test flavonoids dose-dependently inhibited production of $O^{\bullet}$ (Table 1) and HOCl in PMA-stimulated PMN (Table 2). The effect was maximum at a concentration of 100 μM.

It should be emphasized that dihydroquercetin and quercetin in concentrations of 10 and 100 μM were equally potent in suppressing $O^{\bullet}$ generation by PMN from patients with diabetes mellitus and healthy donors. However, activated PMN from patients were more sensitive to the influence of flavonoids in minimum concentration (1 μM). Dihydroquercetin and quercetin in a concentration of 1 μM inhibited $O^{\bullet}$ generation by PMN from patients with diabetes mellitus and healthy donors by 50 and 35%, respectively.

Dihydroquercetin and quercetin dose-dependently suppressed MDA accumulation during LPO induced by Fe^{2+}-ascorbate (Table 2). MDA accumulation in NIDDM patients and healthy donors was blocked by dihydroquercetin and quercetin in a concentration of 100 μM. Dihydroquercetin and quercetin in a concentration of 10 μM reduced the content of MDA in PMN from patients by 25 and 14%, respectively, compared to the control. In healthy donors this parameter decreased by 18 and 14%, respectively.

Published data show that the intensity of $O^{\bullet}$ generation by PMA-stimulated PMN in NIDDM patients surpasses that in healthy donors [6,10]. However, we revealed no differences in the amount of HOCl produced by PMA-stimulated PMN from patients and healthy donors. It was probably associated with decreased activity of myeloperoxidase in PMN from NIDDM patients. We agree with Ya. A. Aleksandrovskii [1] when he said that functional strain of PMN during NIDDM is related to long-term hyperglycemia. This state is associated with increased protein kinase C activity in neutrophils. Protein kinase C activates NADPH oxidase via phosphorylation of its cytosolic fragments. These changes result in high-intensity generation of free oxygen radicals. Previous studies showed that protein kinase C activity increases during oxidative stress induced by hyperglycemia [7]. PMA interacts with protein kinase C and, therefore, initiates receptor signal transduction after the formation of diacylglycerol and inositol triphosphate. In NIDDM patients the intensity of $O^{\bullet}$ generation by PMA-stimulated PMN was higher than in healthy donors. These

TABLE 1. Effects of Quercetin (QV) and Dihydroquercetin (DQV) on Generation of Superoxide Radicals in PMA-Stimulated Human PMN (nmol $O^{\bullet}_{2}$/10^6 PMN, *M*±*m*)

Group		Control	1 μM	10 μM	100 μM
Patients with diabetes mellitus					
	DQV	15.3±0.9*	7.6±0.4**	6.1±0.3**	1.2±0.2**
	QV		9.9±0.3**	6.0±0.5**	1.1±0.3**
Healthy donors					
	DQV	8.6±0.7	6.3±0.6	5.8±0.5**	1.0±0.2**
	QV		6.0±0.4	5.7±0.5**	0.9±0.1**

Note. Here and in Table 2: $p<0.01$: *compared to healthy donors; **compared to the control.

TABLE 2. Formation of Hypochlorous Acid in PMA-Stimulated Human PMN and Accumulation of MDA in the System of Fe^{2+} and Ascorbate under the Influence of QV and DQV (*M*±*m*)

Group, characteristic		Control	1 μM	10 μM	100 μM
Formation of hypochlorous acid, nmol HOCl/10^6 PMN					
patients with diabetes mellitus	DQV	118.3±6.2	109.6±5.4	83.1±4.7	39.2±5.1**
	QV		110.1±6.3	85.3±5.5	41.3±4.3**
healthy donors	DQV	110.0±4.2	109.3±2.6	85.6±2.5	41.0±3.2**
	QV		107.6±2.9	89.7±3.5	40.9±3.8**
Formation of MDA, rel. units/10^6 PMN					
patients with diabetes mellitus	DQV	19.42±2.05*	17.16±2.41	14.56±2.30	2.34±1.12**
	QV		17.32±2.33	16.70±2.53	2.41±1.37**
healthy donors	DQV	12.05±2.73	10.55±2.16	9.88±2.05	2.03±1.32**
	QV		11.16±2.29	10.36±2.42	2.45±1.21**

data suggest that the patients with diabetes mellitus are characterized by high reactivity of the protein kinase C-dependent pathway for activation of NADPH oxidase. It is important that intensive generation of $O^{\bullet}_{2}$ by PMA-stimulated PMN is observed even in patients with compensated diabetes mellitus. Probably, activation of the NADPH oxidase system is realized via other mechanisms.

Activation of NADPH oxidase in neutrophils is related not only to the effect of protein kinase C, but also to other extracellular processes (enhanced release and oxidation of arachidonic acid; and high-intensity mobilization of Ca^{2+} in PMN from NIDDM patients) [5]. Previous studied revealed a Ca^{2+}-independent pathway for signal transduction and activation of the respiratory burst, which is determined by changes in membrane characteristics during activation of phagocytizing cells. Viscosity of the PMN membrane decreases in NIDDM patients [5], which probably makes these cells more susceptible to activation.

Our experiments showed that the amount of LPO products increases in nonactivated and activated PMN from NIDDM patients. The data indicate that PMN in these patients are in activated state. Intensive generation of lipid radicals in membranes of PMN from NIDDM patients can be associated with activation of lipoxygenases and cyclooxygenases that catalyze oxidation of arachidonate and other polyunsaturated fatty acids in cell membranes.

Flavonoids suppress not only generation of $O^{\bullet}_{2}$ and HOCl, but also production of MDA during oxidation of neutrophil membranes.

The antioxidant effect of flavonoids can be associated with not only inhibition of enzymes catalyzing generation of radicals, but also trapping of these radicals [9,15]. Quercetin blocks activation of protein kinase C in PMN. Therefore, this substance prevents PMN activation [4]. Moreover, quercetin inhibits phosphorylation of tyrosine and activation of phospholipase D in activated neutrophils [11]. Quercetin acts as a potent inhibitor of myeloperoxidase (both purified or present in activated PMN). Quercetin can bind HOCl that is formed in the system of myeloperoxidase, H_2O_2, and chlorine [8]. Dihydroquercetin probably decreases activities of protein kinase and myeloperoxidase in activated PMN (similarly to quercetin), which contributes to suppression of free oxygen radical generation in these cells.

Antioxidant activity of flavonoids can be related to binding of transition metals involved in conversion of H_2O_2 into hydroxyl radical (Haber-Weiss reaction). The hydroxyl radical initiates LPO and impairs viscosity and permeability of cell membranes. It was assumed that complexes of flavonoids and metal ions inhibit LPO due to limited access to fatty acids in cell membrane phospholipids [9]. Our results confirm this hypothesis. The test preparations suppressed LPO in PMN membranes induced by Fe^{2+} and ascorbate, *i.e.*, agents initiating free radical oxidation of membrane lipids without involvement of ROS.

Our results show that dihydroquercetin and quercetin *in vitro* decrease functional activity of PMN from NIDDM patients. It cannot be excluded that dihydroquercetin that abolishes activation of PMN would prevent the development of oxidative stress and progression of angiopathies. This compound holds much promise for combination therapy of NIDDM patients.

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