啓明醫大學術誌 第21券 1號
Keimyung Med J
Vol. 21, No. 1, June, 2002

Activities of Antioxidant Enzymes in Neutrophils from Patients with Diabetes Mellitus*

Hung Gwan Lee, M.D. and Kyo Cheol Mun, M.D.

Department of Biochemistry,
Keimyung University School of Medicine, Taegu, Korea

Abstract : To know the mechanism of impaired bactericidal function, especially impaired hydrogen peroxide production, in neutrophils from patients with diabetes mellitus, the activities of antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase were determined in neutrophils from 12 patients with diabetes mellitus. Catalase activity in the neutrophils from the patients with diabetes mellitus were higher than that from healthy controls ($P<0.05$). Glutathione peroxidase activity in the neutrophils was higher without significance. Superoxide dismutase activity in the neutrophils was lower without significance. These results suggest that the production of hydrogen peroxide exerting a direct germicidal ability is impaired in patients with diabetes mellitus due to the decrease in dismutation of superoxide radicals by superoxide dismutase and the increase in dismutation of hydrogen peroxide by catalase and glutathione peroxidase, and these alterations might affect microbicidal activity of neutrophils in patients with diabetes mellitus.

Key Words : Neutrophils, Catalase, Glutathione peroxidase, Superoxide dismutase

Introduction

When neutrophils engulf bacteria, they exhibit a rapid increase in oxygen consumption known as the respiratory burst[1-3]. This phenomenon reflects the rapid utilization of oxygen and production from it of large amounts of reactive derivatives, such as superoxide anion, hydrogen peroxide, hydroxyl radical, and hypochlorite ion[1-3]. Some of these products are potent microbicidal agents[1-3]. Any superoxide that enters the cytosol of the phagocytic cell is converted to hydrogen peroxide by the action of superoxide dismutase[1-3]. And hydrogen peroxide is disposed of by the action of catalase and glutathione peroxidase[1-3]. Like these,

* The present research has been conducted by the Grant of Graduate School, Keimyung University, Taegu, Korea in 2001.

several enzymes are directly linked to the fate of the highly reactive oxygen metabolites during phagocytosis.

There will be some alterations in the activities of antioxidant enzymes because it is known that diabetics are prone to infection due to impaired neutrophil function[4] and impaired hydrogen peroxide production[5].

To know the mechanism of impaired bactericidal function, especially impaired hydrogen peroxide production, in neutrophils from patients with diabetes mellitus, the activities of three antioxidant enzymes were measured in neutrophils from 12 patients with diabetes mellitus.

Materials and methods

1. Patients

Experiments were performed in 12 patients with non-insulin dependent diabetes mellitus. Twelve normal healthy subjects matched for age and sex were used as controls. Peripheral venous blood was drawn into test tubes containing heparin.

2. Chemicals

Tris (hydroxymethyl) aminomethane, dimethylsulfoxide, catalase, superoxide dismutase, glutathione peroxidase, NADPH, hydrogen peroxide, cytochrome c, reduced glutathione, glutathione reductase were purchased from Sigma (USA). dextran T-500 was purchased from Pharmacia Biotech (Sweden). All other chemicals were of the highest purity commercially available.

3. Neutrophil preparation

Neutrophils from diabetes mellitus and healthy donors were prepared from fresh whole human blood. Neutrophils were separated according to method of Clark and Nauseef[6]. Blood was mixed with an equal volume of 3% dextran T-500 in normal saline solution, and incubated in upright position for 20 min at room temperature. Supernatant was aspirated carefully, and centrifuged at 250 × g for 10 min. Cell pellet was resuspended in a volume of 0.9% NaCl equal to the starting volume of blood. 10 ml Ficoll-Hypaque solution was layered beneath the cell suspension. And centrifuged for 40 min at 400 × g. The top layer as well as the Ficoll-Hypaque layer was aspirated. Residual RBC was removed by hypotonic lysis with 20 mL of cold 0.2% NaCl for exactly 30 sec. Isotonicity was restored by adding 1.8% NaCl solution. Separated neutrophils were homogenized twice for 10 sec in ice-cold phosphate buffered saline before enzyme assay. The entire operation was carried out in the cold (0-4℃).

4. Enzyme assays

Superoxide dismutase was assayed using alkaline dimethylsulfoxide as a superoxide anion-generating system in association with cytochrome c as a superoxide anion-indicating scavenger by the method of Hyland *et al.*[7]. 200 μL of 7.5×10^6 neutrophils/mL was added to 1 mL of 0.2 M

potassium phosphate buffer (pH 8.6) containing 10^{-4} M EDTA and 2×10^{-5} M cytochrome c. Tubes were kept in an ice bath for 20 min. Then, 0.5 mL alkaline dimethylsulfoxide which contains 1% water and 5 mM NaOH, was added with stirring. Absorbance of reduced cytochrome c was determined at 550 nm against samples prepared under same conditions except that dimethylsulfoxide did not contain NaOH. One unit of its activity was defined as the amount which inhibited the reduction of cytochrome c by 50%.

Catalase activity was assayed using hydrogen peroxide as a substrate by the method of Kim *et al.*[8]. At 25℃, the decrease in absorbance was measured at 240 nm for 1 min after adding 20 μL of 10^7 neutrophils/mL into the 3.0 mL of 50 mM phoshate buffer, pH 7.0, containing 5 mM H_2O_2 and 0.1 mM EDTA. One unit of catalase was defined as equivalent to the elimination of 1 μM of H_2O_2 per min under the above condition.

Glutathione peroxidase activity was assayed by the method of St Clair and Chow[9] in which the oxidation of glutathione is coupled to the glutathione reductase, thus promoting consumption of NADPH. 100 μL of 10^7 neutrophils/mL was added to 875 μL of the reagent solution which contains 2 mM EDTA, 1 mM sodium azide, 1 mM reduced glutathione, 0.2 mM NADPH and 100 units of glutathione reductase in 50 mM Tris-HCl buffer, pH 7.6. 25 μL of hydrogen peroxide was added to start the reaction, and the final concentration of hydrogen peroxide was 0.25 mM. The amount of NADPH oxidized was recorded spectrophotometically at 340 nm.

5. Statistics

Values were expressed as mean ± SD. Statistical evaluation of significant difference between means was performed with the Student's t-test. P values less than 0.05 were considered significant.

Results

Superoxide dismutase activity in the neutrophils from the patients with diabetes mellitus was 1.52 ±0.71 unit/10^7cells/min. Its activity was lower in diabetes mellitus than in normal control group, but it was not significant (Fig. 1).

Catalase activity in the neutrophils from the patients with diabetes mellitus was 70.20 ± 19.54 μmol hydrogen peroxide destroyed/10^7cells/min. Its activity was significantly higher in diabetes mellitus than in normal control group (Fig. 2).

Glutathione peroxidase in the neu-

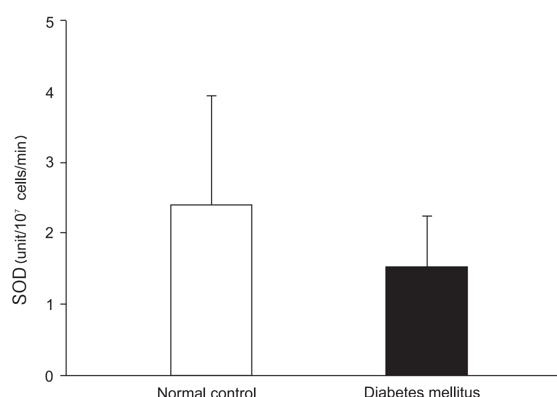

Fig. 1. Superoxide dismutase (SOD) activity in neutrophils from patients with diabetes mellitus. SOD was assayed using alkaline dimethylsulfoxide as a superoxide anion-generating system in association with cytochrome c as a superoxide anion-indicating scavenger.

trophils from the patients with diabetes mellitus was 63.29 ± 23.77 nmol NADPH oxidized/10^7cells/min. Its activity was higher in diabetes mellitus than in normal control group, but it was not significant (Fig. 3).

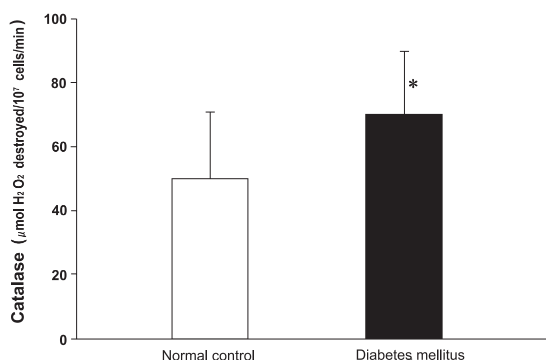

Fig. 2. Catalase activity in neutrophils from patients with diabetes mellitus. Catalase activity was assayed using hydrogen peroxide as a substrate. *: P<0.05.

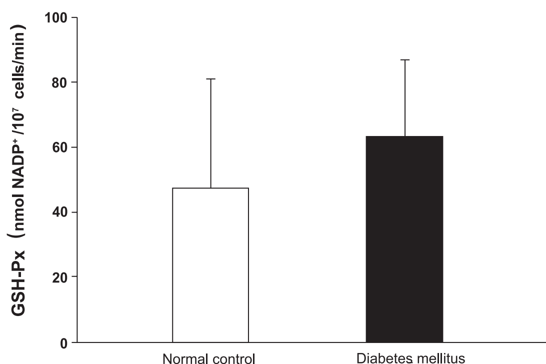

Fig. 3. Glutathione peroxidase (GSH-Px) activity in neutrophils from patients with diabetes mellitus. GSH-Px activity was assayed by the spectrophotometric method using glutathione reductase and NADPH.

Discussion

Oxygen free radicals can be defined as oxygen molecules or molecular fragments that have an unpaired electron[10-11]. They are formed in all living organisms during physiological and pathophysiological metabolism and cause cell and tissue damages due to their high chemical reactivity[10-11].

Neutrophils are part of the professional phagocytes which undergo a 'respiratory burst': in this process, large quantities of oxygen are drawn into the cells[1-3]. The consumed oxygen is used in the formation of superoxide anion, hydroxyl radical and hydrogen peroxide, which have been implicated in the killing of invading bacteria by the neutrophils[1-3]. During this unique process, several antioxidant enzymes like catalase, superoxide dismutase and glutathione peroxidase are directly linked to the fate of the highly reactive oxygen metabolites[1-3].

The purpose of the present study was to examine the activity of three intracellular antioxidant enzymes i.e. catalase, superoxide dismutase and glutathione peroxidase, involved in neutrophil oxidative bactericidal activity, as a measure of phagocytosis associated burst of oxidative metabolism for bactericidal purpose.

The results of the present study demonstrate that neutrophils from diabetes mellitus patients have altered antioxidant enzymatic activity. While superoxide dismutase activity in the neutrophils from patients with diabetes mellitus were inhibited (Fig. 1), both catalase and glutathione peroxidase activities were augmented (Fig. 2 & 3). These enzymes are involved oxidative bactericidal ability in neutrophils. They are linked to the production, fate and reactivity of the free radicals and other intermediates of oxygen

reduction, as part of the respiratory burst[1-3]. Superoxide dismutase is responsible for the dismutation of superoxide anion to hydrogen peroxide, which is the main product of the respiratory burst exerting a direct bactericidal ability[1-3], and catalase and glutathione peroxidase are responsible to the dissociation of hydrogen peroxide[1-3]. The results of this experiment suggest that the production of hydrogen peroxide exerting a direct germicidal ability is impaired in the patients with diabetes mellitus, as reported by Inoue *et al.*[5], due to the decrease in dismutation of superoxide radicals by superoxide dismutase and the increase in dismutation of hydrogen peroxide by catalase and glutathione peroxidase. This impairment in antioxidant enzymes activity, involved in the respiratory burst and phagocytosis, may contribute to the understanding of the impaired bactericidal function in neutrophils found in these patients.

Summary

To know the mechanism of impaired bactericidal function, especially impaired hydrogen peroxide production, in neutrophils from patients with diabetes mellitus, the activities of antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase were determined in neutrophils from 12 patients with diabetes mellitus. Catalase activity in the neutrophils from the patients with diabetes mellitus were higher than that from healthy controls ($P<0.05$). Glutathione peroxidase activity in the neutrophils was higher without significance. Superoxide dismutase activity in the neutrophils was lower without significance. These results suggest that the production of hydrogen peroxide exerting a direct germicidal ability is impaired in patients with diabetes mellitus due to the decrease in dismutation of superoxide radicals by superoxide dismutase and the increase in dismutation of hydrogen peroxide by catalase and glutathione peroxidase, and these alterations might affect microbicidal activity of neutrophils in patients with diabetes mellitus.

References

1. Halliwell B, Gutteridge JMC. Free radicals as useful species. In: Halliwell B, Gutteridge JMC, editors. *Free Radicals in Biology and Medicine.* Oxford: Clarendon Press; 1989, p.366-415.
2. Babior BM, Benna JE, Chanock SJ, Smith RM. The NADPH oxidase of leukocytes: the respiratory burst oxidase. In: Scandalios JG, editor. *Oxidative Stress and the Molecular Biology of Antioxidant Defenses.* New York: Cold Spring Harbor Laboratory Press; 1997, p.737-83.
3. Murray RK. Red and white blood cells. In: Murray RK, Granner DK, Mayer PA, Rodwell VW, editors. *Harper's Biochemistry.* 25th ed. London: Appleton & Lange; 2000, p.763-79.
4. Ortmeyer J, Mohsenin V. Inhibition of phospholipase D and superoxide generation by glucose in diabetic neutrophils. *Life Sci* 1996; **59**(3): 255-62.
5. Inoue S, Lan Y, Muran J, Tsuji M. Reduced hydrogen peroxide production in neutrophils from patients with diabetes. *Diabetes Res Clin Pract* 1996; **33**(2): 119-27.
6. Clark RA, Nauseef WM. Preparation and functional

analysis of human nonlymphoid cells. In: Coligan JE, Kruisbeek AM, Margulies DH, Shevach EM, Strober W, editors. *Current Protocols in Immunology.* Washington DC: National Institutes of Health; 1993, Vol **1**. Suppl 6. p. 7.23.1-17.

7. Hyland K, Voisin E, Banoun H, Auclair C. Superoxide dismutase assay using alkaline dimethylsulfoxide as superoxide anion-generation system. *Anal Biochem* 1983; **135**(2): 280-7.
8. Kim MC, Lee BR, Koh K. Calcium and NADH potentiate the superoxide radical production in renal ischemia/reflow of rats. *Korean J Biochem* 1993; **25**: 1-8.
9. St Clair DK, Chow CK. Glutathione peroxidase. Activity and steady-state level of mRNA. In: Punchard NA, Kelly FJ, editors. *Free Radicals. A practical approach.* Oxford: Oxford University Press; 1996, p.1-8.
10. Moslen M. Reactive oxygen species in normal physiology, cell injury and phagocytosis. In: Armstrong D, editor. *Free Radicals in Diagnostic Medicine. A Systems Approach to Laboratory Technology, Clinical Correlations, and Antioxidant Therapy.* New York: Plenum Press; 1994, p.17-27.
11. Punchard NA, Kelly FJ. Introduction. In: Punchard NA, Kelly FJ, editors. *Free Radicals. A Practical Approach.* Oxford: Oxford University Press; 1996, p.1-8.