Effect of Tolrestat, an Aldose Reductase Inhibitor, on Neutrophil Respiratory Burst Activity in Diabetic Patients

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One hypothesis for the reduction in oxidative killing of neutrophils in diabetic patients is that increased polyol pathway activity during hyperglycemia reduces intracellular levels of nicotinamide adenine dinucleotide phosphate (NADPH), resulting in the reduction of neutrophil superoxide production during the respiratory burst. To test this hypothesis, we assessed the effect of tolrestat, an aldose reductase inhibitor, on neutrophil respiratory burst activity (NRBA) in diabetic patients. We measured fasting plasma glucose (FPG), hemoglobin A_1 (HbA_1), and NRBA levels in 79 diabetic patients and 48 normal controls. NRBA was reassessed in 34 patients after 4 weeks of tolrestat or placebo treatment, in seven controls after 4 weeks of tolrestat treatment, and in seven patients after 4 weeks of blood glucose control. NRBA was determined by flow cytometry, which detected fluorescent 2′,7′-dichlorofluorescein (DCF) in neutrophils formed from 2′,7′-dichlorofluorescein diacetate (DCF-DA) during phorbol myristate acetate (PMA)-induced respiratory bursts. Diabetic patients showed lower NRBA than the normal controls (mean cellular fluorescence, 438 ± 103 *v* 668 ± 101, mean ± SD, $P < .001$). NRBA in diabetic patients showed a negative correlation with HbA_1 ($r = -.336$, $P < .005$). Tolrestat treatment for 4 weeks in 17 patients restored the reduced NRBA to an almost normal level (relative NRBA, 0.55 ± 0.20 *v* 0.99 ± 0.36, $P < .05$) despite the fact that FPG level did not change (11.8 ± 2.8 *v* 11.4 ± 2.8 mmol/L). NRBA of these patients after tolrestat treatment was not significantly different from that of seven control subjects treated with tolrestat for 4 weeks. In 17 placebo-treated patients, there were no significant changes in NRBA and FPG level. The vigorous blood glucose control for 4 weeks in seven patients (16.6 ± 2.1 *v* 8.6 ± 2.3 mmol/L) also restored the reduced NRBA to almost normal (relative NRBA, 0.55 ± 0.21 *v* 0.90 ± 0.30, $P < .05$). The result that the reduced NRBA in diabetic patients was restored to almost normal either by tolrestat treatment or by blood glucose control strongly supports the hypothesis of this study.

A GROWING BODY OF EVIDENCE is consistent with the hypothesis that metabolic imbalances associated with hyperglycemia are of central importance in the pathogenesis of late complications of diabetes.[1] Although a unifying explanation for the association of diabetes and infectious complications has not appeared, hyperglycemia also serves as a pivotal factor in the predisposition of diabetic patients to infection by impairing the basic mechanisms of host defense.[2] Disturbances of neutrophil function including abnormal adherence, chemotaxis, phagocytosis, and intracellular killing of bacteria have been observed in diabetic patients.[3-10] Among these, defects in intracellular killing are the most convincing and reproducible abnormalities and are aggravated by worsening metabolic control.

The initial step of intracellular oxidative killing in neutrophils involves respiratory bursts in which molecular oxygen is converted to superoxide by a nicotinamide adenine dinucleotide phosphate (NADPH)-dependent membrane oxidase. The pentose phosphate pathway (PPP) produces the NADPH required for this process.[11] One hypothesis for the reduction in oxidative killing of neutrophils in diabetic patients is that increased polyol pathway activity during hyperglycemia, converting glucose to sorbitol by NADPH-dependent aldose reductase, reduces intracellular levels of NADPH, resulting in the reduction of neutrophil superoxide production during the respiratory burst.[12-16] In addition to several in vitro studies suggesting that increased polyol pathway activity inhibits neutrophil function,[12-15] a recent report that impaired neutrophil killing of *Escherichia coli* in diabetic patients was abolished by in vivo administration of an aldose reductase inhibitor supports this hypothesis.[16] However, there has been no study in which the effect of in vivo aldose reductase inhibition on neutrophil respiratory burst activity (NRBA) was directly assessed.

In this study, we assessed the effect of tolrestat, an aldose reductase inhibitor, on NRBA in diabetic patients. We also assessed the effect of blood glucose control in diabetic patients on NRBA. NRBA was measured by flow cytometry, which detected fluorescent 2′,7′-dichlorofluorescein (DCF) in neutrophils formed from 2′,7′-dichlorofluorescein diacetate (DCF-DA) during phorbol myristate acetate (PMA)-induced respiratory bursts.[17]

SUBJECTS AND METHODS

Patients

A total of 79 diabetic patients (36 men and 43 women aged 55 ± 13 years) were recruited from the diabetic clinics at Hallym University Hospitals, Seoul, Korea. Control blood samples were obtained from 48 healthy nondiabetic subjects (22 men and 26 women aged 53 ± 14 years). All subjects were free of renal insufficiency and any detectable infectious disease at the time of study. They were not taking any drugs other than oral hypoglycemic agents (OHA) or insulin.

Study Design

Fasting plasma glucose (FPG), hemoglobin A_1 (HbA_1), and NRBA were measured in 79 diabetic patients, and NRBA in the patients was compared with that in 48 normal controls. Among diabetic patients, 34 were randomly selected and alternately randomly assigned by order of recruitment to tolrestat or placebo, and either a 200-mg tolrestat capsule (Alredase, manufactured by Geonil Pharmaceutical, Korea, under license of Wyeth-Ayerst Labs, New York, NY) or an identical placebo

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Submitted February 14, 1996; accepted January 6, 1997.

Supported by a research grant from Hallym Medical Center.

Presented in part in abstract form in Diabetes 44:154A, 1995 (suppl 1).

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0026-0495/97/4606-0006$03.00/0

Table 1. Clinical and Demographic Characteristics of the Patients

Characteristic	Total Patients (n = 79)	Tolrestat Group (n = 17)	Placebo Group (n = 17)	Glucose-Control Group (n = 7)
Age (yr)*	55 ± 13	54 ± 13	52 ± 14	51 ± 15
Sex (M/F)	36/43	7/10	7/10	4/3
Duration of diabetes (yr)*	6.6 ± 5.8	7.6 ± 6.3	7.1 ± 5.3	7.9 ± 5.5
Type of diabetes (IDDM/NIDDM)	8/71	3/14	2/15	1/6
Current therapy (diet/OHA/insulin)	2/45/32	0/7/10	0/6/11	0/5/2
Diabetic retinopathy (−/+)	57/22	12/5	13/4	6/1
Diabetic neuropathy (−/+)	52/27	11/6	12/5	6/1
FPG (mmol/L)*	12.0 ± 3.1	11.8 ± 2.8	11.3 ± 2.9	16.6 ± 2.1
HbA_1 (%)*	10.6 ± 2.9	9.9 ± 2.1	9.5 ± 2.2	11.5 ± 1.0

Abbreviations: IDDM, insulin-dependent diabetes mellitus; NIDDM, non–insulin-dependent diabetes mellitus.
*Mean ± SD.

capsule was administered daily in the morning for 4 weeks. Seven normal controls also received a 200-mg tolrestat capsule daily in the morning for 4 weeks. At the end of the treatment period, FPG and NRBA were reassessed. In another seven diabetic patients showing poor blood glucose control (initial FPG >14 mmol/L), FPG and NRBA were reassessed after 4 weeks of blood glucose control with increased dosages of OHA or insulin. Patient characteristics are shown in Table 1. The study was approved by the hospital ethics committee, and informed consent was obtained from all of the diabetic patients.

Measurement of Glucose and HbA_1

Plasma glucose concentration was measured with a glucose oxidase analyzer (Hitachi 736-20 autoanalyzer; Wako, Osaka, Japan). HbA_1 level was measured electrophoretically using a commercial kit (Glytrac; Corning, Palo Alto, CA).

Measurement of NRBA

Venous blood from the subjects was placed in an EDTA tube and processed within 2 hours.[17] Fourteen microliters of 100-mmol/L DCF-DA (Eastman Kodak, Rochester, NY) was added to 700 μL EDTA whole blood in a tube wrapped with aluminum foil, and the mixture was preincubated in a shaking water bath for 15 minutes at 37°C. Then, 10 μL 500-mmol/L sodium azide (Sigma, St Louis, MO) was added and incubated for 3 minutes. To the aliquots of 100 μL of this mixture, 10 μL phosphate-buffered saline (PBS) or 10 μL 1.1-ng/mL or 3.3-ng/mL PMA (Sigma) was added to a final concentration of 0, 100, and 300 ng/mL PMA and incubated in a shaking water bath for 15 minutes at 37°C. Then, 2 mL cold erythrocyte lysis solution (Becton Dickinson, Sunnyvale, CA) was added and incubated for 5 minutes at room temperature. After centrifugation at 250× *g* for 5 minutes at 4°C, each cell pellet was washed with PBS, resuspended in 1 mL 1% paraformaldehyde, and kept at 4°C for 20 minutes.

Intracellular DCF fluorescence was determined by flow cytometry (FACS; Becton Dickinson). Neutrophils were distinguished from the other leukocytes by flow cytometric measurements of cellular light-scatter, and their DCF fluorescence was gated to separate histograms. NRBA was expressed as mean cellular fluorescence.[17-19] At least 10,000 cells were examined in each sample. The relative NRBA of diabetic patients was calculated by dividing NRBA of a patient by NRBA of a control measured at the same time. Control blood samples for relative NRBA were obtained from one of three nondiabetic subjects.

Statistical Analysis

Data are expressed as the mean ± SD. Selected comparisons were performed with Student's unpaired or paired *t* test and a Wilcoxon signed-rank sum test. Linear regression analysis determined relations between variables of interest. *P* less than .05 was considered statistically significant.

RESULTS

NRBA of Controls and Diabetic Patients

NRBA was increased in response to PMA in a dose-dependent manner in both normal controls and diabetic patients (Fig 1). NRBA of diabetic patients in the absence of PMA was slightly higher than that of normal controls (111 ± 29 *v* 94 ± 21, $P < .05$). NRBA of diabetic patients was lower than that of normal controls in the presence of 100 ng/mL PMA (137 ± 45 *v* 192 ± 77, $P < .005$) and 300 ng/mL PMA (438 ± 103 *v* 668 ± 101, $P < .001$). NRBA of the three controls for relative NRBA was 98 ± 8 in the absence of PMA and 676 ± 45 with 300 ng/mL PMA (n = 38 measurements). The relative NRBA of diabetic patients showed a negative correlation with HbA_1 ($r = -.336$, $P < .005$; Fig 2), but showed no correlation with FPG.

Effect of Tolrestat Treatment on NRBA

Tolrestat treatment for 4 weeks in 17 patients restored the reduced NRBA to almost normal (relative NRBA with 100 ng/mL PMA, 0.66 ± 0.21 *v* 1.17 ± 0.49, $P < .01$; relative NRBA with 300 ng/mL PMA, 0.55 ± 0.20 *v* 0.99 ± 0.36, $P < .05$) despite that their FPG levels did not change significantly (11.8 ± 2.8 *v* 11.4 ± 2.8 mmol/L; Fig 3). NRBA of these patients after tolrestat treatment was not significantly different from that of seven control subjects treated with tolrestat for 4 weeks (NRBA with 100 ng/mL PMA, 210 ± 81 *v* 186 ± 51; NRBA with 300 ng/mL PMA, 629 ± 12 *v* 681 ± 83). In the 17 placebo-treated patients, there were no significant changes in NRBA (relative NRBA with 100 ng/mL PMA, 0.58 ± 0.22 *v* 0.51 ± 0.29; relative NRBA with 300 ng/mL PMA, 0.53 ±

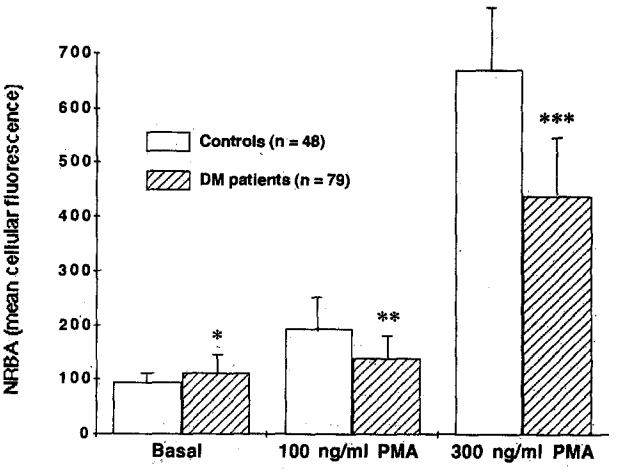

Fig 1. NRBA in controls and diabetic patients (mean ± SD). *$P < .05$, **$P < .005$, and ***$P < .001$ *v* controls.

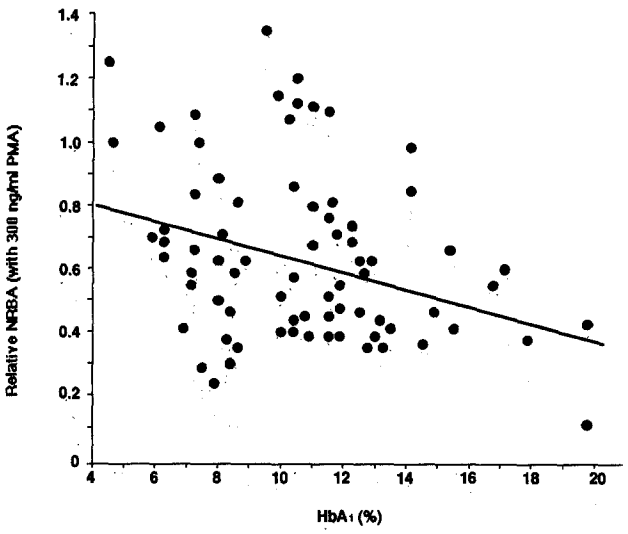

Fig 2. Relationship between relative NRBA with 300 ng/mL PMA and HbA_1 in diabetic patients ($r = -.336$, $P < .005$).

0.21 *v* 0.55 ± 0.21) and FPG level (11.3 ± 2.9 *v* 11.4 ± 2.8 mmol/L).

Effect of Blood Glucose Control on NRBA

The vigorous blood glucose control for 4 weeks in seven patients (from 16.6 ± 2.1 to 8.6 ± 2.3 mmol/L) who initially showed poor blood glucose control also restored the reduced NRBA to almost normal (relative NRBA with 100 ng/mL PMA, 0.65 ± 0.24 *v* 0.99 ± 0.23, $P < .05$; relative NRBA with 300 ng/mL PMA, 0.55 ± 0.21 *v* 0.90 ± 0.30, $P < .05$; Fig 4).

DISCUSSION

The respiratory burst of polymorphonuclear leukocytes generating active oxygen derivatives is essential for intracellular killing of certain ingested bacteria. The oxidative burst of neutrophils can be monitored by assays of the oxygen consumption, chemiluminescence, formation of redox reaction products, and generation of reactive oxygen species.[8-11] In most in vitro studies including luminol-dependent chemiluminescence and ferricytochrome *c* reduction techniques, a large number of purified and washed neutrophils are required to monitor generation of active oxygen derivatives. A major disadvantage with these methods is that a large amount of blood is required to isolate sufficient neutrophils. In addition, isolation of neutrophils is a time-consuming and laborious procedure and may result in some cellular damage. In this study, the oxidative burst expressed in the generation of hydrogen peroxide in stimulated neutrophils was quantitatively monitored in a small amount of whole blood using DCF-DA, PMA, and flow cytometry.[17] This method is based on the oxidation of nonfluorescent DCF-DA to a fluorescent DCF by hydrogen peroxide and peroxidase. The fluorescent DCF compound, being polar, cannot diffuse outside the cell. Therefore, it is possible to detect DCF-DA oxidation of individual neutrophils using single-cell analysis by flow cytometry. PMA, a soluble stimulus for the oxidative burst, provides a useful tool for studying the respiratory burst of neutrophils irrespective of phagocytosis.[11] NRBA expressed as mean DCF fluorescence per cell was increased by PMA in a dose-dependent manner in both normal controls and diabetic patients (Fig 1).

It has been observed that in vitro high concentrations of glucose inhibited neutrophil killing of *Candida albicans*, and this inhibition appeared to be associated with reduced superoxide production of neutrophils.[8,13,14] In the present study, neutrophils of diabetic patients showed lower NRBA than those of normal controls in response to PMA (Fig 1). The observation of impaired NRBA in diabetic patients is consistent with a previous report that superoxide production by leukocytes in response to PMA, measured by a Luminol-dependent chemiluminescence and ferricytochrome *c* reduction technique, was significantly reduced in diabetes mellitus.[9]

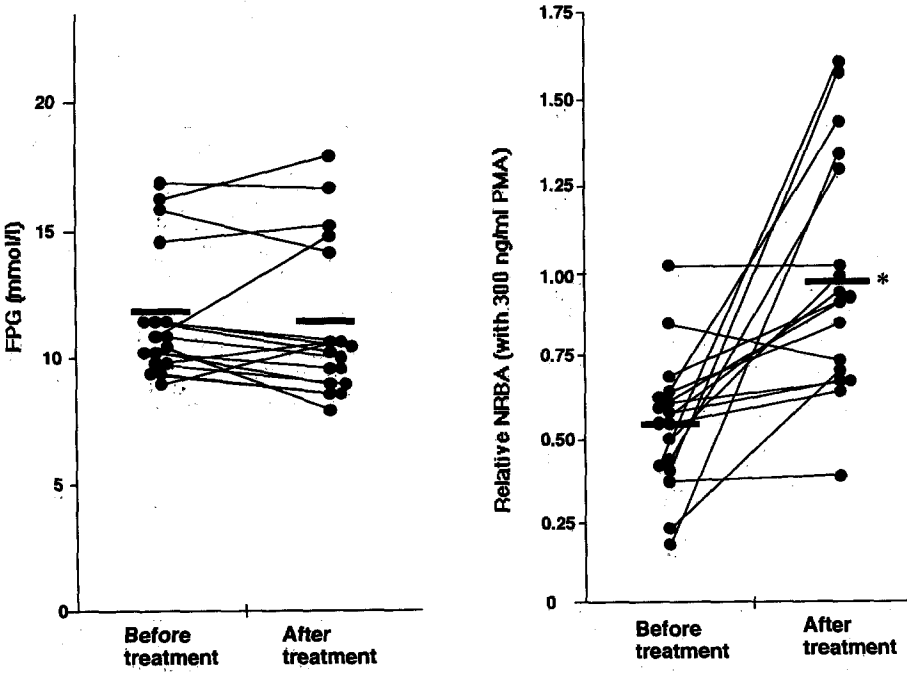

Fig 3. FPG and relative NRBA (with 300 ng/mL PMA) before and after tolrestat treatment for 4 weeks. The bar indicates the mean value. *$P < .05$ *v* before tolrestat treatment.

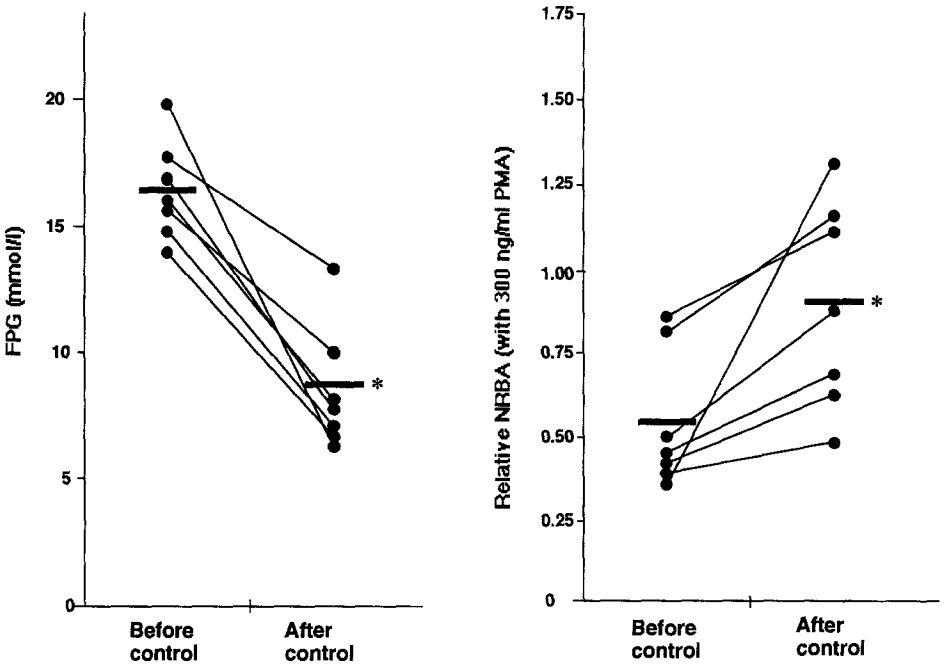

Fig 4. FPG and relative NRBA (with 300 ng/mL PMA) before and after blood glucose control for 4 weeks. The bar indicates the mean value. *$P < .05$ *v* before blood glucose control.

Tolrestat treatment for 4 weeks in 17 patients in the present study restored the reduced NRBA to almost normal despite the fact that FPG levels did not change. Placebo treatment for the same duration in another 17 patients did not change NRBA significantly. Although we could not measure how much nonfluorescent DCF-DA entered the polymorphonuclear cells, tolrestat seems to have no direct effect on DCF-DA uptake by these cells, considering that in normal controls NRBA was not significantly changed after tolrestat treatment for 4 weeks. These data suggest that increased aldose reductase activity might be responsible for the suppression of NRBA in diabetic patients. Under normoglycemic conditions, glucose is phosphorylated by hexokinase to glucose-6-phosphate, which is then metabolized by glycolysis or the PPP. During hyperglycemia, hexokinase (K_m, ~5 mmol/L) becomes saturated, and it is suggested that in neutrophils, like many other tissues that do not require insulin for glucose transport into the cell, glucose enters the polyol pathway and is metabolized to sorbitol by NADPH-dependent aldose reductase.[20-22] Neutrophils in this study were protected by tolrestat treatment for 4 weeks throughout their life span from bone marrow to blood, so that damage to them by hyperglycemia through the polyol pathway might have been prevented. Thus, the increased polyol pathway activity during hyperglycemia could result in the decrease of neutrophil superoxide production during the respiratory burst, possibly through the reduction of intracellular NADPH levels.

The impaired NRBA observed in patients with diabetes was increased to almost normal levels not only by administering the aldose reductase inhibitor but also by restoring a normal blood glucose level. This finding strongly supports the hypothesis that increased polyol pathway activity during hyperglycemia is responsible for decreased NRBA in diabetic patients. Studies on the effect of aldose reductase inhibitor on neutrophil NADPH content are required to further support the hypothesis. The results of this study suggest that aldose reductase inhibitors may have a role in enhancing neutrophil function in the presence of infection in poorly controlled diabetic patients, in addition to rapid and strict blood glucose control and administration of appropriate antibiotics.

NRBA of diabetic patients in the basal state was slightly higher than that of normal controls, suggesting that diabetic neutrophils are under weak stimulation in the absence of any normal stimulus such as phagocytosis. This might result in ineffective bactericidal activity or contribute to tissue damage in diabetic vascular complications. These possibilities have not been studied yet.

From our hypothesis, a correlation between the degree of hyperglycemia and NRBA of diabetic patients is expected. However, NRBA of diabetic patients showed a weak negative correlation with HbA_1 ($r = -.336$, $P < .005$) and showed no correlation with FPG (Fig 2). This finding is consistent with previous studies that found no correlation or only a weak correlation between FPG or glycated hemoglobins and decreased bactericidal activity of neutrophils.[14-16] This might have resulted from a small number of study subjects or a low sensitivity of the methods for neutrophil function measurements. In addition, glucose concentrations on the day of measurement or for the previous 3 months may not be well reflected in NRBA, because the neutrophil's life span is 8 to 17 days. Fructosamine levels, which provide an estimate of diabetic control for the preceding 1 to 2 weeks, might have shown better correlation with NRBA than FPG or HbA_1 did.

In conclusion, our study demonstrates that reduced NRBA in diabetic patients could be restored to almost normal either by tolrestat treatment or by blood glucose control. These results strongly support the hypothesis that increased polyol pathway activity during hyperglycemia results in the decrease of neutrophil superoxide production during the respiratory burst, possibly through the reduction in intracellular levels of NADPH. Further studies including direct measurement of neutrophil NADPH content are required to elucidate the exact mechanisms.

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