Neutrophils play an important role during the early host response to infection by a coordinated series of effector functions that include chemotaxis, phagocytosis, and the generation of reactive oxygen species (1, 2). Phagocytic leukocytes play a critical role in immunological protection against infections by recognizing, digesting, and killing microorganisms (1). It has been reported that during phagocytosis there is activation of phosphatidylinositol cascade, leading to inositoltrisphosphate (IP3) and diacylglycerol (DAG) production (2). The diacylglycerol is an activator of protein kinase C (PKC). All these processes are Ca2+-dependent, as intracellular Ca2+ is a second messenger in many signal transduction pathways (3-5). Several reports indicate that intracellular Ca2+ also is involved in insulin signaling (5-7). The role of cytosolic calcium in insulin signaling is likely to rely on localized changes in intracellular Ca2+ rather than large Ca2+ swings mediated by influx from extracellular compartments (8, 9).

**INTRODUCTION**

Neutrophils play an important role during the early host response to infection by a coordinated series of effector functions that include chemotaxis, phagocytosis, and the generation of reactive oxygen species (1, 2). Phagocytic leukocytes play a critical role in immunological protection against infections by recognizing, digesting, and killing microorganisms (1). It has been reported that during phagocytosis there is activation of phosphatidylinositol cascade, leading to inositoltrisphosphate (IP3) and diacylglycerol (DAG) production (2). The diacylglycerol is an activator of protein kinase C (PKC). All these processes are Ca2+-dependent, as intracellular Ca2+ is a second messenger in many signal transduction pathways (3-5). Several reports indicate that intracellular Ca2+ also is involved in insulin signaling (5-7). The role of cytosolic calcium in insulin signaling is likely to rely on localized changes in intracellular Ca2+ rather than large Ca2+ swings mediated by influx from extracellular compartments (8, 9).

**Kinetics of Calcium Ion Concentration Accompanying Transduction of Signals into Neutrophils from Diabetic Patients and Its Modification by Insulin**

The goal of the study was to evaluate the process of Ca2+-mediated transduction of signals into neutrophils from patients with type I diabetes and its modification by insulin. The study was performed with the use of isolated peripheral blood neutrophils from 20 diabetic patients and 30 healthy volunteers. Isolated granulocytes were stimulated separately by fMLP or insulin, or by both substances added to the medium in combinations: fMLP + insulin (after 20 min) or insulin + fMLP (after 20 min). fMLP evoked fast intracellular increase of free Ca2+ concentration in neutrophils compared with the resting state (P<0.001). Similarly, the peak of fluorescence, as measured by Fluo 3 to Fura Red ratio, was significantly higher in neutrophils stimulated by insulin. Insulin did not cause any changes in intracellular Ca2+ level when it was added to the previously fMLP-stimulated cells. Prestimulation with insulin significantly decreased fMLP-induced intracellular free Ca2+ concentration, expressed as Fluo3/Fura Red ratio compared with fMLP alone (1.77 ± 0.6 vs. 2.63 ± 0.8, P<0.001). No relation between initial intracellular Ca2+ in the resting state and the response to insulin was found. Nor was the response to fMLP alone related to intracellular Ca2+ before stimulation. A strong correlation was observed between initial intracellular Ca2+ after incubation with insulin and the response to fMLP (r=0.90, P<0.0001). In diabetic granulocytes, the intracellular Ca2+ was significantly lower than in those from healthy donors in unstimulated cells (P<0.001), after iMLP stimulation (P<0.0001), in medium enriched by insulin (P<0.05), and after fMLP stimulation in insulin rich medium (P<0.001). Only in fMLP prestimulated samples, the emission of light did not differ after stimulation with insulin in granulocytes from both diabetic and healthy subjects. In conclusion, patients with type 1 diabetes have decreased levels of cytosolic Ca2+ after insulin and fMLP stimulation in polymorphonuclear granulocytes. This abnormality is probably primarily responsible for the impaired neutrophilic function seen in these patients.

**Key words:** diabetes, granulocytes, insulin, intracellular calcium
MATERIAL AND METHODS

All enrolled subjects gave informed consent for participation in the study. The Ethics Committee of Warsaw Medical University in Warsaw, Poland approved the study protocol.

Blood samples were collected from 20 patients with type I diabetes, aged 22-57 (the mean age of 41 ± 3 yr). The mean time from the diagnosis of diabetes type I was 17 ± 3 yr. Patients with hypertension were excluded from the study. None of the patients received any medication except for the long-term insulin therapy. As a control, blood samples were collected from 30 healthy donors free from any metabolic and immunologically mediated disorder, aged 19-45 (the mean of 30 ± 7 yr).

Neutrophils

Three milliliters of venous blood were taken from the ulnar vein to a tube containing heparin (10 U/ml). The blood count was determined using a Coulter HMX analyzer. Neutrophils were identified microscopically in the blood smear after hematological staining. Neutrophils were isolated by 25 min centrifugation at 1200 x g on Gradisol G with d = 1.115 ± 0.002 g/cm 3 (POLFA, Lodz, Poland) mixed with Histopaque in proportion (3/2). After isolation and final washing, the cells were pelleted and resuspended in RPMI-1610 medium (Sigma Chemicals St. Luis, MO). Ninety five percent of the cells had the morphology of neutrophils. To the suspension of neutrophils, 5 µM of Fluo 3 and Fura Red (Molecular Probes, Eugene, OR) were added and the aliquots were incubated in dark at 37°C. Then, the cells were washed and resuspended in RPMI-1610 at a concentration of 2 mln/ml and divided into three aliquots.

Flow cytometry

Neutrophils were discriminated by flow cytometric measurements of cellular forward angle and right angle scatter, using an FC500 flow cytometer (Beckman Coulter, Hialeah, FL), according the method described earlier (16). Fluo 3 and Fura Red were excited at 488 nm, Fluo 3 emission was detected at 515-535 nm and Fura Red emission was detected at 665-685 nm. The first 40 s of the analysis was considered as an initial, resting state. Then, the measurement was interrupted to add stimulants, after which it was continued for the next 60 s. fMLP at concentration of 10^-6 M (Sigma Chemicals St. Luis, MO) and insulin at a concentration of 20 pmol/l (Bioton, Warsaw, Poland) were used as the stimulants. The ratio intensity of Fluo3/Fura Red vs. time was also calculated.

Statistical analysis

Statistical analysis was performed with the use of Statistica 6.0. Each result was calculated as a mean ±SD. Results were compared with the use of the non-parametric Mann-Whitney U test. The Spearman test was used for an analysis of correlations between different parameters. A P value <0.05 was considered statistically significant.

RESULTS

Isolated granulocytes were stimulated by fMLP or insulin alone, or by both added to the medium in the following combinations: fMLP+insulin or insulin+fMLP, both after 20 min. As a control, unstimulated cells were used.

fMLP evoked fast intracellular increase of free Ca²⁺ in neutrophils compared with the resting state Fluo3 emission of light significantly increased (P<0.001) and Fura Red emission of light decreased (P<0.05). As a consequence the Fluo3/Fura Red ratio increased significantly (P<0.0001) compared with control. Similarly, the peak of fluorescence, as measured by the ratio, Fig. 1. Fluorescence of granulocytes from diabetic patients after fMLP stimulation with and without addition of insulin. The results are expressed as a percentage change of the fluorescence from Fluo 3, Fura Red, and the Fluo3/Fura Red index in relation to the control (granulocytes in the control medium).

![Flow cytometry](image)

Table 1. Fluorescence in granulocytes from diabetic patients after fMLP and/or insulin stimulation (n=20).

<table>
<thead>
<tr>
<th>Fluorescent dye</th>
<th>Control</th>
<th>fMLP</th>
<th>Insulin</th>
<th>Insulin + fMLP</th>
<th>fMLP + Insulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluo 3</td>
<td>14.5 ±1.7</td>
<td>39.9±3.9</td>
<td>18.5±3.1</td>
<td>33.3±2.6</td>
<td>20.9±14.7</td>
</tr>
<tr>
<td>Fura Red</td>
<td>23.4±1.9</td>
<td>15.2±2.0</td>
<td>24.3±2.1</td>
<td>18.8±2.1</td>
<td>23.9±2.7</td>
</tr>
<tr>
<td>Fluo 3/Fura Red</td>
<td>0.62±0.2</td>
<td>2.63±0.8</td>
<td>0.76±0.2</td>
<td>1.77±0.6</td>
<td>0.87±0.3</td>
</tr>
</tbody>
</table>

Fig. 2. Correlation between initial intracellular Ca²⁺ level in granulocytes from diabetic patients after incubation with insulin, as measured by the Fluo 3/Fura Red ratio, and the response to fMLP of insulin preincubated cells (n=20, P<0.0001). Axis X and Y show the percentage changes in Fluo3/Fura Red index in samples with insulin addition and insulin plus fMLP.
It is suggested that albuminuria in type 2 diabetic patients is associated with a primary defect in intracellular Ca2+ homeostasis (18). The present study shows an appreciable impairment of Ca2+ kinetics in neutrophils from diabetics. We have recently reported on neutrophils obtained from healthy volunteers that the effects of insulin at the cellular level are related to cytosolic Ca2+ changes (19). That supports the hypothesis that insulin is an interactive factor in Ca2+ disturbances which, therefore, in diabetic patients could be secondary to disregulated insulin secretion or its exogenous administration. Insulin is known as an important regulator of a variety of biological effects including growth, development, and metabolism (20). The molecular mechanisms of its action have been intensively studied by various approaches (21, 22). Intracellular signaling pathways are working downstream of the insulin-dependent signals and have to do with intracellular Ca2+ changes (23). The scenario for an early intracellular signal transduction involving activation of protein kinase cascade is well documented. Phospholipase C (PLC) catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate to produce the second messengers IP3 and DAG. IP3 binds to its intracellular receptor, a ligand-gated calcium channel located in the endoplasmic reticulum, and triggers the release of Ca2+ from internal stores, leading to a rapid and transient increase in intracellular Ca2+, whereas DAG stimulates members of the PKC and PKD families (24-26). Calcium is a versatile intracellular messenger that is used throughout the life cycle of an organism to control diverse biological processes (26). It has been suggested that diabetes and cardiovascular disease are linked to a common defect of divergent cation metabolism, including calcium (27). Decreased response to fMLP has been described in type II diabetes (28). We now demonstrate that similar impairment is also present in type I diabetes and it is directly linked to a decrease in cytosolic Ca2+. These observations are in accord with the findings that recurrent respiratory tract infections in children are connected to decreased response to fMLP (9). Thus, decreased intracellular Ca2+ level in immune cells may be one of the mechanisms of impaired immunity in diabetic patients. We also demonstrate in the present study a strong correlation between intracellular Ca2+ level after incubation with insulin and the intensity of the response to fMLP. It may indicate that insulin restores an impaired response of phagocytic cells to bacterial stimulant in insulin deficient individuals. There are similar observations of other authors concerning fibroblasts (29). The corollary is that a correct response of granulocytes to extrinsic stimuli depends on the proper function of cell signaling pathways. A reduction in neutrophilic functional activity may contribute to higher susceptibility to, and severity of, infections in diabetes mellitus. Such alterations are strongly connected to cytosolic calcium disturbances. We conclude that patients with type I diabetes have a decreased cytosolic calcium level in polymorphonuclear granulocytes after insulin and fMLP stimulation. This abnormality is probably responsible for impaired neutrophilic function seen in these patients.

Conflict of interests: None declared.

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