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Effect of acute hyperglycemia and/or hyperinsulinemia on polymorphonuclear functions in healthy subjects

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Abstract

Abnormal immune functions of polymorphonuclear (PMN) cells occur in a variety of pathophysiological conditions. There exists a close link between glucose metabolism and PMN functions. The aim of this study was to assess the effect of short-term hyperglycemia and/or hyperinsulinemia on phagocytosis and respiratory burst of PMN cells in healthy subjects in vivo. The study was performed on 12 healthy subjects (mean age, 26.9 ± 1.6 years; body mass index, 24.4 ± 0.84 kg/m^2). Acute hyperglycemia and/or hyperinsulinemia was induced by three 4-hour-long clamp studies—hyperglycemic hyperinsulinemic clamp (HHC), hyperinsulinemic euglycemic clamp (HEC), and isolated hyperglycemic clamp with insulin secretion blockade (HGC). Polymorphonuclear cell phagocytosis and PMN cell respiratory burst (mean percentage and mean fluorescent intensity of phagocyting/activated PMN cells, phagocytic, and respiratory burst indexes) were evaluated by flow cytometry under basal and stimulated conditions. Results detected during clamp studies were compared with those found during a control study with saline infusion. Significant reductions in the mean percentage of phagocyting cells measured under basal conditions after the HHC (6.7% ± 1.3% vs 12.1% ± 4.3%; $P < .05$) and HGC (4.5% ± 1.8% vs 9.9% ± 2.1%; $P < .05$) were found in comparison with the preclamp study period; however, these results did not differ significantly from those detected during the control clamp (CC) study. Significantly higher phagocytic (115.1 ± 65 vs 35.8 ± 18.6; $P < .05$) and respiratory burst indexes (16.5 ± 3 vs 10.1 ± 1.4; $P < .05$) measured under basal conditions were found after HEC in comparison with the preclamp data. However, these data did not differ significantly from those found after the CC study. No significant differences in other parameters of detected PMN cell immune functions were found after HHC, HEC, and HGC. In conclusion, immune functions of PMN cells were not significantly influenced by short-lasting hyperglycemia and/or hyperinsulinemia induced in vivo by clamp techniques in healthy subjects compared to changes induced by the CC study. Further studies on the short-term effect of glucose metabolism on PMN functions in diabetic patients should be considered necessary.

1. Introduction

Phagocytosis by polymorphonuclear (PMN) cells constitutes an essential tool of host defense against bacterial and fungal infections. The phagocytic process can be separated into several major steps: chemotaxis, attachment of particles to the cell surface of phagocytes, ingestion, and intracellular killing by oxygen-dependent and -independent mechanisms [1,2]. These PMN functions require energy derived from glucose [3]. There may exist close links between glucose metabolism and PMN functions based on metabolic and pathophysiological conditions.

The oxygen-dependent intracellular killing of infectious agents in PMN cells, known as the respiratory burst, consists of a sequence of metabolic events activated after contact of foreign particles with the PMN membrane. The respiratory burst includes the generation of reactive oxygen intermediates (ROI) [4-7]. The generation of ROI relates to the oxidation of glucose via the hexose-monophosphate shunt. The microbicidal activity of activated macrophages against certain intracellular and extracellular pathogens is correlated with their production of ROI [8,9].

Abnormal phagocytosis occurs in a variety of clinical disorders and may be associated with the neutrophil itself or with immunoglobulin or complement defects [6,10-17]. In

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diabetic patients, a 50% reduction in PMN glucose use and glycolysis was found [18]; however, a significant increase in glucose use and glycogen formation was observed in leukocytes after treatment of diabetic patients with insulin [18,19]. Some studies showed functional PMN cell abnormalities such as the impairment of chemotaxis [20,21], adherence [22], and ingestion [20,23], which were associated with chronic hyperglycemia [20,23,24]. The respiratory burst of stimulated PMN cells was significantly reduced in both type 1 [23,25] and type 2 diabetic patients [26] with poor metabolic control. It is unclear how the acute changes in short-lasting high glucose and insulin serum levels could impact the potential for infection in a clinical setting.

The effect of acute hyperglycemia on PMN functions was still predominantly assessed during in vitro studies. There were described significant changes in immune functions up to high glucose medium levels. Reduced PMN functions such as phagocytosis and respiratory burst with reduced superoxide production were shown in several reports [27-29].

To date, the influence of hyperinsulinemia on PMN cell activities was assessed *very rarely* in vivo [18,30]. Nevertheless, the effect of acute hyperglycemia on PMN functions has not been studied yet.

The aim of our study was to assess the effect of combined short-lasting hyperglycemia and hyperinsulinemia, isolated hyperinsulinemia, and isolated hyperglycemia on phagocytosis and respiratory burst of PMN cells in healthy subjects during clamp techniques. These models of conditions correspond far more with the pathophysiological status in patients with diabetes mellitus.

2. Materials and methods

2.1. Subject characteristics

Twelve healthy subjects without any concomitant disease were included into the study. They had a mean age of 26.9 ± 1.6 years and body mass index of 24.4 ± 0.84 kg/m^2. Each of the healthy subjects had to have normal blood counts, normal glucose tolerance according to the 1999 World Health Organization diagnostic criteria [31] (mean fasting glycemia, 4.6 ± 0.12 mmol/L), and normal glycosylated hemoglobin (mean HbA_{1c}, 4.8% ± 0.09%). Normal glucose tolerance was confirmed by an oral glucose tolerance test. None of the study subjects took medications known to influence plasma glucose or insulin concentrations or had pathological levels of erythrocyte sedimentation rate and routine biochemical laboratory tests (creatinine, urea, electrolytes, lipid concentrations, urine analysis). Immune defects were excluded by laboratory tests of physiological parameters related to immune functions.

The exclusion criteria included respiratory, renal, hepatic, gastrointestinal, immune, infectious or other diseases, alcoholism, and drug abuse. Acute and chronic diseases were excluded based on medical history, physical examination, and routine laboratory tests of blood counts, biochemical parameters, etc. None had a defined immune defect and a history of acute infectious disease during the last 3 months.

All subjects provided written informed consent before participation in this study, which was reviewed and approved by the local ethics committee.

2.2. Experimental design

All subjects were examined after an overnight fast. They were instructed to adhere to their ordinary lifestyle and avoid alcohol consumption, strenuous exercise, and major changes in food intake for 3 days before the clamp study. The study procedures in each healthy subject consisted of 4 consecutive clamp studies, each of 4 hours duration performed in 14-day intervals.

2.2.1. Study 1

The hyperglycemic hyperinsulinemic clamp (HHC) study was performed as described previously [32,33]. Briefly, a new Teflon cannula (Venflon; Ohmeda, Helsingborg, Sweden) was inserted into an antecubital vein for the infusion of all test substances. A second cannula (Venflon; Becton-Dickinson, Helsingborg, Sweden) was inserted retrogradely into a wrist vein for blood sampling, and the hand was placed in a warming device to achieve venous blood arterialization. The aim of the HHC was to raise the plasma glucose concentration acutely to a fixed hyperglycemic plateau of 17 mmol/L and to maintain it at that level for 4 hours. The high plasma glucose level was achieved with a bolus of 40% glucose in a previously calculated dose (dose [mL] = weight [kg] × [17 − plasma glucose concentration] × 0.0855). Thereafter, blood glucose levels were measured in blood samples obtained at 5- or 10-minute intervals. Plasma glucose concentrations throughout the study periods were maintained at the hyperglycemic level by a continuous 15% glucose infusion at variable rates adjusted according to glucose levels.

Blood samples for immunoreactive insulin (IRI) determination were taken before (0 minutes) and at 60, 120, 180, 210, 225, and 240 minutes of each clamp study. Blood counts, parameters of phagocytosis, and respiratory burst of PMN cells were determined from blood samples taken at 0 and 240 minutes of each clamp study.

2.2.2. Study 2

The hyperinsulinemic euglycemic clamp (HEC) procedure was described previously [32-34]. A primed constant insulin infusion (1 mU/kg per minute of Actrapid HM; NovoNordisk, Copenhagen, Denmark) was administrated to raise and maintain the plasma insulin concentration at approximately 60 mU/L. The whole blood glucose levels were measured in blood samples obtained at 5- or 10-minute intervals. Glycemia was maintained at fasting level (4-6 mmol/L) by a continuous infusion of 15% glucose at variable rates adjusted according to glucose measurements. Addition of saline was infused to match the liquid volume applied during the HHC in study 1.

2.2.3. Study 3

To test the effect of isolated hyperglycemia in vivo, the isolated hyperglycemic clamp (HGC) with insulin secretion blockade was performed. A hyperglycemic plateau of 17 mmol/L was achieved with a 40% glucose bolus in a previously calculated dose (dose [mL] = weight [kg] × [17 − glycemic level] × 0.0855) followed by a continuous intravenous 15% glucose infusion at variable rates adjusted according to glucose level measurements as in study 1. The endocrine insulin secretion was blocked by continuous somatostatin infusion at the rate of 500 μg/h (somatostatin injection power, 1 × 3 mg + solvens; UCB SA Pharma, Braine, L'Alleut, Belgium). To ensure time and volume compatibility with the HHC, the rest of the liquid volume applied throughout the first clamp study was substituted by an adequate amount of drinking water.

2.2.4. Study 4

The control clamp (CC) study consisted of infusion of saline and drinking of water time- and volume-matched to the HHC.

The mean plasma glucose concentration during study 1 was 17 ± 0.35 mmol/L with coefficients of variation lower than 5%. Because of the physiological stimulation of endogenous insulin secretion, the mean reactive plasma insulin concentration was 352.3 ± 66 μU/mL. Euglycemia (mean plasma glucose concentration, 4.7 ± 0.09 mmol/L with coefficients of variation <5%) and hyperinsulinemia (mean plasma insulin concentration, 57 ± 5 μU/mL) were maintained throughout study 2. Stable hyperglycemia (mean plasma glucose concentration, 16.4 ± 0.7 mmol/L with coefficients of variation <5%) was achieved in study 3 with endocrine insulin secretion blockade by somatostatin, resulting in a mean plasma insulin value of 23.8 ± 3.6 μU/mL. During the CC study (study 4), the mean plasma glucose concentration and mean plasma insulin value were 4.8 ± 0.12 mmol/L with coefficients of variation lower than 5% and 3.4 ± 0.5 μU/mL, respectively.

2.3. Analytical methods

The plasma glucose concentrations were measured on a Beckman Analyzer (Beckman Instruments, Fullerton, CA) using the glucose oxidase method. HbA_{1c} was evaluated by the Bio-Rad Haemoglobin A_{1c} Column Test (Bio-Rad Laboratories, Munich, Germany). Immunoreactive insulin was determined by radioimmunoassay using Immunotech Insulin IRMA kit (Immunotech, Prague, Czech Republic).

Phagocytosis of PMN cells was evaluated by flow cytometry using a commercially available quantitative test (Phagotest; Orpegen Pharma, Heidelberg, Germany). Phagocytosis of PMN cells was analyzed as described previously [35,36]. Phagotest measures the overall percentage of granulocytes showing phagocytosis—the ingestion of one or more bacteria per cell—as well as the individual cellular phagocytic activity (number of bacteria per cell).

One half of a heparinized whole-blood sample (100 μL per test) was incubated in an ice bath for 10 minutes as a negative control sample. The second half of the blood sample was incubated with fluorescein-labeled opsonized (with immunoglobulin and complement) bacteria *Escherichia coli* (*E coli* FITC opsonized 1×10^9 bacteria per milliliter) in a water bath at 37°C for 10 minutes. At the end of the incubation time, phagocytosis was stopped by placing all samples on ice and adding 100 μL of quenching solution. After washing with the washing solution, the erythrocytes were disrupted by adding 2 mL of the lysing solution to the blood samples. Finally, 200 μL of the staining solution was added to the samples, and the percent of the phagocyting cells, as well as the intensity of the fluorescence per cell, was evaluated as described below.

Respiratory burst of PMN cells was evaluated by flow cytometry using a commercially available test (Bursttest; Orpegen Pharma, Heidelberg, Germany). The test measures the percentage and the enzymatic activity of the ROI-producing PMN cells. Bursttest uses unlabelled opsonized *E coli* (a particulate stimulants) for PMN stimulation. *Escherichia coli* was chosen as a more physiological stimulants with closer relation to infection.

Respiratory burst of the PMN cells was analyzed as described previously [35,36]. Briefly, mixed heparinized blood (100 μL per test) was incubated in an ice bath for 10 minutes to cool the blood samples to 0°C. Half of the whole blood was mixed with 20 μL of precooled *E coli*. The nonstimulated sample served as a negative background control. Next, 20 μL of washing solution was added. After mixing, the samples were incubated in a water bath at 37°C for 10 minutes. After that, 20 μL of substrate solution (dihydrorhodamine 123; DHR123) was added and incubated for another 10 minutes at 37°C. At the end of the incubation, the blood samples were lysed and fixed with 2 mL lysing solution, then washed and supplemented with 200 μL DNA staining solution. Measurements of the cell suspensions were done using the flow cytometer, as described below, within 30 minutes after 10 minutes of incubation with the staining solution.

Comparisons of blood count changes found after each clamp study with those detected during the CC study showed significantly reduced amounts of leukocytes after the HHC (Δ leukocytes after the HHC vs after the CC, 1.17 vs 0.19; $P < .05$) and after the HGC (Δ leukocytes after the HGC vs after the CC, 1.12 vs 0.19; $P < .05$). These results did not influence the percentage and mean fluorescent intensity of phagocyting and active PMN cells measured by flow cytometry, as approximately 10 000 PMN cells from each blood sample were taken to perform the measurement of activity of PMN phagocytosis and respiratory burst.

2.4. Analysis of PMN functions by flow cytometry

Percentage of phagocytic PMN cells, mean fluorescent intensity of the labeled phagocyting PMN cells, and percentage and mean fluorescent intensity of cells with

Table 1
The phagocytosis of PMN cells measured under basal conditions before and after HHC, HEC, isolated HGC, and CC studies

A. Basal phagocytic parameters before and after individual clamp studies

Phagocytic parameters	Type of clamp							
	HHC		HEC		HGC		CC	
	Before clamp (n = 12)	After clamp (n = 12)	Before clamp (n = 12)	After clamp (n = 12)	Before clamp (n = 12)	After clamp (n = 12)	Before clamp (n = 12)	After clamp (n = 12)
MP of phagocyting PMN cells (%)	12.1 ± 4.4	6.7 ± 1.3*	7.3 ± 1.7	7.5 ± 1.5	9.9 ± 2.1	4.5 ± 1.8*	7.8 ± 2.2	5.8 ± 1.8
MFI of phagocyting PMN cells	389 ± 121	392 ± 70	214 ± 122	341 ± 152	177 ± 98	167 ± 93	418 ± 142	427 ± 139
Phagocytic indexes	14.6 ± 1.2	13.2 ± 1.1	35.8 ± 18.6	115.1 ± 65*	65.5 ± 40.6	65 ± 40.8	176 ± 67.3	122.5 ± 37

B. A comparison of the changes in phagocytic parameters assessed during clamp studies with those found during the CC study

Changes of parameters	Type of clamp			
	HHC	HEC	HGC	CC
ΔMP of phagocyting PMN cells	−5.4 ± 4	0.27 ± 1.8	−5.1 ± 2	−1.1 ± 2.3
ΔMFI of phagocyting PMN cells	2.84 ± 96	126.5 ± 150	3.3 ± 6	−27 ± 151
ΔPhagocytic indexes	1.5 ± 0.83	−87.2 ± 68.8	0.1 ± 0.7	18.5 ± 32

Results are means ± SE. MFI indicates mean fluorescent intensity; MP, mean percentage; Δ, change in parameters before and after the clamp study.
* $P < .05$; statistically significant changes after HHC and HGC clamps in comparison with parameters found before HHC and HGC clamps, respectively; other parameters are not significant.

oxidative burst were measured in the blood samples with the use of the FACSCalibur (Becton-Dickinson, San Jose, CA) flow cytometer equipped with a laser beam emitting 488 nm of blue-green light. For the analyses of blood leukocytes from the samples (at least 10 000 cells per sample), the CellOuest software (BDIS, CA) was used. The fluorescence was estimated using histograms from the FL2 channel. The power of the study was less than 80%.

2.5. Data analysis

Polymorphonuclear functions (phagocytosis and respiratory burst) under basal conditions (activity of negative samples) and after *E coli* stimulation in the pre- and postclamp periods were compared. Phagocytic and respiratory burst indexes related to PMN functions were calculated (mean fluorescent intensity of phagocyting or active PMN

Table 2
The phagocytosis of PMN cells measured after *E coli* stimulation before and after HHC, HEC, isolated HGC, and CC studies

A. Stimulated phagocytic parameters before and after individual clamp studies

Stimulated parameters	Type of clamp							
	HHC		HEC		HGC		CC	
	Before clamp (n = 12)	After clamp (n = 12)	Before clamp (n = 12)	After clamp (n = 12)	Before clamp (n = 12)	After clamp (n = 12)	Before clamp (n = 12)	After clamp (n = 12)
MP of phagocyting PMN cells (%)	81.9 ± 3.9	81.6 ± 4.5	59.4 ± 9.6	56.4 ± 7.7	66 ± 8.8	69.5 ± 4.5	60 ± 6.8	57.3 ± 7.4
MFI of phagocyting PMN cells	1268 ± 214	1333 ± 247	1018 ± 219	638 ± 196	1159 ± 445	653 ± 215	1129 ± 198	1240 ± 145
Phagocytic indexes	104.4 ± 28.8	78.7 ± 21.7	362 ± 98	349.5 ± 92	451 ± 87	429 ± 38.2	417 ± 84.2	410.3 ± 81.1

B. A comparison of the changes in phagocytic parameters assessed during clamp studies with those found during the CC study

Changes in parameters	Type of clamp			
	HHC	HEC	HGC	CC
ΔMP of phagocyting PMN cells	−0.3 ± 1.1	4.8 ± 5	−3.1 ± 0.5	−2.4 ± 1.7
ΔMFI of phagocyting PMN cells	65.4 ± 113	−156 ± 57	−193.8 ± 27	−26.9 ± 59
ΔPhagocytic indexes	31.1 ± 41.6	16.6 ± 11.1	21.3 ± 48.1	13.4 ± 40.7

Results are means ± SE. No parameters are significant.

Table 3
The respiratory burst of PMN cells measured under basal conditions before and after HHC, HEC, isolated HGC, and CC studies

A. Basal parameters of respiratory burst before and after individual clamp studies

Basal parameters	Type of clamp							
	HHC		HEC		HGC		CC	
	Before clamp (n = 12)	After clamp (n = 12)	Before clamp (n = 12)	After clamp (n = 12)	Before clamp (n = 12)	After clamp (n = 12)	Before clamp (n = 12)	After clamp (n = 12)
MP of active PMN cells (%)	4.5 ± 1.4	2.5 ± 0.4	2.9 ± 0.7	2.1 ± 0.6	3.9 ± 1	6.8 ± 2.6	3.4 ± 1.1	3.1 ± 1.1
MFI of active PMN cells	288 ± 53	300 ± 66	501 ± 95	678 ± 156	475 ± 116	494 ± 173	516 ± 128	684 ± 175
Respiratory burst indexes	14.6 ± 1.2	13.2 ± 1.1	10.1 ± 1.4	16.5 ± 3*	14.6 ± 3.1	17.3 ± 3.5	10.1 ± 1.3	11.9 ± 2.3

B. A comparison of the changes in respiratory burst parameters assessed during clamp studies with those found during the CC study

Changes in parameters	Type of clamp			
	HHC	HEC	HGC	CC
ΔMP of active PMN cells	−2 ± 1.4	−0.8 ± 0.7	2.8 ± 1.7	−0.3 ± 1
ΔMFI of active PMN cells	12.1 ± 60	177.5 ± 104	6.6 ± 92	168.2 ± 106
ΔRespiratory burst indexes	1.7 ± 0.9	−5.0 ± 3.3	0.7 ± 3.8	−2.9 ± 2.1

Results are means ± SE. No parameters are significant.

cells divided by mean fluorescent intensity of nonphagocyting or nonactive PMN cells). The HHC, HIC, and HGC studies were also compared with the time- and volume-controlled saline infusion.

The statistical data analysis was performed by the BMDP PC 90 statistical software (Los Angeles, CA). The comparison of data and indexes evaluated before and after each clamp study was performed by paired Wilcoxon test in each healthy subject, and the comparison of data changes among clamp studies was performed by Kruskal-Wallis test in each healthy subject. The Spearman rank correlation coefficient was used to determine any significant correlation between assessed data. All data are expressed as means ± SE. All results including indexes were also analyzed after logarithmic transformation of non-Gaussian distributed data. P values of less than .05 were considered significant.

3. Results

We found significant reductions in the mean percentage of phagocyting PMN cells measured under basal conditions after the HHC ($P < .05$) and after the HGC ($P < .05$;

Table 4
The respiratory burst of PMN cells measured after *E coli* stimulation before and after HHC, HEC, isolated HGC, and CC studies

A. Stimulated parameters of respiratory burst before and after individual clamp studies

Stimulated parameters	Type of clamp							
	HHC		HEC		HGC		CC	
	Before clamp (n = 12)	After clamp (n = 12)	Before clamp (n = 12)	After clamp (n = 12)	Before clamp (n = 12)	After clamp (n = 12)	Before clamp (n = 12)	After clamp (n = 12)
MP of active PMN cells (%)	89.6 ± 5.3	89.8 ± 4.8	91.1 ± 3.3	79.4 ± 8.9	82.6 ± 7	91.2 ± 2.7	90 ± 3.5	89.4 ± 2.5
MFI of active PMN cells	2850 ± 508	2620 ± 476	2487 ± 353	2407 ± 307	1906 ± 358	1662 ± 287	3045 ± 344	2886 ± 393
Respiratory burst indexes	104.4 ± 28.8	78.7 ± 21.7	52.9 ± 25.4	45 ± 14.5	36.9 ± 14.8	38.3 ± 14.2	37 ± 9.6	40.1 ± 12.5

B. A comparison of respiratory burst parameters assessed during clamp studies with those found during the CC study

Changes in parameters	Type of clamp			
	HHC	HEC	HGC	CC
ΔMP of active PMN cells	0.7 ± 4.4	−11.7 ± 8.8	10.3 ± 7.1	−0.5 ± 2.4
ΔMFI of active PMN cells	−107 ± 212	−80.3 ± 83	−238 ± 198	−158.5 ± 389
ΔRespiratory burst indexes	29.7 ± 35.3	−1.8 ± 21.5	1.3 ± 9.1	−7.1 ± 9.4

Results are means ± SE. No parameters are significant.

Table 1A). Other phagocytic parameters of PMN cells assessed after individual clamp studies under basal conditions were not significantly different from those found before the clamp studies (Table 1A). However, the changes in basal phagocytic PMN parameters evaluated during individual clamp studies did not differ significantly from those of the CC study (Table 1B).

Other parameters of phagocyting PMN cells measured after *E coli* stimulation (Table 2A) were not influenced by acutely induced hyperglycemia and/or hyperinsulinemia during provided clamp studies. Similar to differences in basal PMN phagocytic parameters, the changes in PMN phagocytic parameters before and at the end of the HHC, HEC, HGC, and the CC studies measured after *E coli* stimulation were not statistically different as shown in Table 2B.

The measurements of respiratory burst of PMN cells under basal conditions before and after each clamp study did not differ significantly (Table 3A); their respective changes during the HHC, HEC, and HGC were not significantly different from the results of the CC study (Table 3B). The mean percentage and mean fluorescent intensity of active PMN cells measured after *E coli* stimulation before and after each clamp study did not differ significantly (Table 4A), and their differences under stimulated conditions were not statistically different either from those detected during CC (Table 4B).

Significantly higher phagocytic indexes and respiratory burst indexes evaluated under basal conditions were found after the HEC ($P < .05$). However, these indexes were not significantly different from those found after the CC study (Tables 1 and 3).

All indexes elucidating the activity of phagocytosis and respiratory burst measured after *E coli* stimulation were not statistically different before and after each study clamp. Moreover, their comparison with indexes detected during CC did not differ significantly, even after logistic transformation (Tables 2 and 4).

4. Discussion

The aim of our study was to determine whether short-lasting hyperglycemia and/or hyperinsulinemia induced under defined conditions during clamp studies could influence the immune functions of PMN cells in healthy subjects in vivo. The design of our study was based on theoretical pathophysiological presumptions and on results of previous experimental in vitro studies. To the best of our knowledge, studies performing under exactly defined conditions during clamp studies have not been published yet.

Based on pathophysiological aspects, we presumed a prevailing negative effect of hyperglycemia on phagocytosis and especially on respiratory burst of PMN cells. There exist some hypotheses of pathophysiological action of higher glucose and insulin concentrations on metabolism and activity of PMN cells. They are, in some aspects, controversial.

Physiologically, superoxide radicals are formed during the respiratory burst from oxygen and nicotinamide adenine dinucleotide phosphate (NADPH) molecules under catalyzation of NADPH oxidase [6]. Normally, 40% of glucose is metabolized through hexose monophosphate shunt in PMN cells. Hyperglycemia could induce the several-fold increase in glucose amounts metabolized via hexose monophosphate shunt. Reactive oxygen intermediate production increases proportionally with NADPH production increase [37]. Conversely, during severe hyperglycemia, saturation of hexokinase enzyme could appear. Afterwards, glucose is converted to sorbitol by an NADPH-dependent aldose reductase enzyme in the polyol pathway, in which NADPH is consumed by the aldose reductase enzyme and therefore ROI production decreases [17].

Based on experimental in vitro studies performed on PMN cells from healthy subjects after short-term exposition to hyperglycemia, we suspected various impairments of PMN functions. Cendoroglo et al [27] proved that incubation of PMN cells from healthy subjects in media with high glucose concentrations, comparable to concentrations in dialysis fluids, inhibited phagocytosis and respiratory burst after 30 minutes of incubation. An inhibition of phagocytosis and unaffected activity of respiratory burst (irrespective of glucose concentration or fluid osmolality) after exposition of PMN cells from healthy subjects to high glucose concentrations (25 mmol/L) and hyperosmolarity of peritoneal fluids has been shown by Liberek et al [38]. Nielson and Hindson [39] showed an impairment of respiratory burst of preincubated PMN cells from healthy subjects in dependence on increasing glucose concentration (11-56 mmol/L) during 30 minutes of incubation in vitro.

In our study, we have not found any significant effect of acute short-lasting hyperglycemia (17 mmol/L) on PMN functions, neither on phagocytosis or on respiratory burst of PMN cells. Compensatory effect of both the abovementioned pathophysiological mechanisms participating in ROI production might be involved.

Similarly to hyperglycemic conditions, we did not find any significant effect of short-lasting hyperinsulinemia on immune functions of PMN cells in healthy subjects.

Based on previously reported studies, we supposed that insulin could activate the main PMN functions through specific binding of insulin to its PMN membrane receptor [40,41]. Moreover, as described earlier, insulin was able to increase in vitro glucose consumption of PMN cells from nondiabetic human subjects without influencing the permeability of the plasma membrane to glucose [42]. Spagnoli et al [43] confirmed the activation of PMN functions induced by high insulin concentrations. On the other hand, Hu et al [44] showed that insulin attenuated PMN respiratory burst.

Similar to our results, Safronova et al [40] showed that insulin did not change the basal PMN production of ROI after 60 minutes of PMN cell incubation in different insulin concentrations in the medium. However, the authors reported that insulin could modulate the stimulated respiratory burst

parameters via tyrosine phosphorylation in dependence on temperature and pretreatment of PMN cells with insulin.

A recent study performed by Walrand et al [18] assessed, as in our clamp study, the effect of hyperinsulinemia on PMN cells from healthy subjects in vivo. In contrast to our study, increased chemotaxis, phagocytosis, and bactericidal capacities were found after 4 hours of HEC in healthy subjects [18]. We also found significantly higher phagocytic and respiratory burst indexes after HEC; however, these data did not differ from those detected after the CC study. The differences between Walrand's study and ours consisted in the lower plasma insulin levels used during Walrand's study in comparison with our HEC (46 vs 60 mU/L) and in the different methods assessing PMN functions. In contrast to our study, Walrand et al did not perform a comparison of detected data with data evaluated after the CC study with saline infusion; the effect of time and volume expansion was not excluded. *Escherichia coli*, which serves as a stimulant in our study, is more physiological than formyl-methionyl-leucyl-phenylalanine (fMLP) and phorbol 12–myristate 13–acetate (PMA) used in Walrand's study [18].

There are many differences between studies performed in vivo evaluating the effect of short-lasting hyperglycemia and/or hyperinsulinemia and experimental in vitro studies. Experimental in vitro studies used considerably higher glucose concentrations (11-56 mmol/L) for testing, in comparison with high glucose levels typical in human medicine. In our study, we tested a high glucose serum level (17 mmol/L), which is more realistic in clinical practice. Moreover, the evaluations performed in vitro do not correspond to physiological regulation mechanisms compensating for the effect of acute hyperglycemia and hyperinsulinemia on PMN functions in humans such as mechanisms mediated by secondary messengers, that is, intracellular calcium, tyrosine kinase, enzymes of hexosomonophosphate shunt, scavenger system represented by, for example, superoxidedismutase and at least by cytokines.

Limitations of our study include variations of detected parameters of phagocytosis and respiratory burst, which have been corrected by adequate statistical methods. We cannot exclude less evident changes in PMN functions with short-term changes in glucose or insulin, which can be detectable in more study subjects. The degree of PMN function decreased unambiguously, which is considered as being of clinical importance as it occurs very rarely. It is represented by chronic granulomatous disease, lazy-leukocyte syndrome, myeloperoxidase deficiency, deficiency of leukocyte glucose-6-phosphate dehydrogenase, and by genetically transferred metabolic diseases. All mentioned diseases are demonstrated usually by repeated serious infections and have obvious pathological results based on Phagotest and Bursttest. Such abnormalities were not found in any of the volunteers from our clamp study [45].

In summary, we did not find any significant alteration of phagocytosis and respiratory burst of PMN cells in the healthy subjects during acutely induced short-lasting hyperglycemia and/or hyperinsulinemia in vivo in comparison with the CC with saline infusion. Although there were found elevated phagocytic and respiratory burst indexes after short-lasting isolated hyperinsulinemia and reduced percentages of phagocyting PMN cells after short-lasting hyperglycemia and hyperinsulinemia or isolated hyperglycemia measured under basal conditions, these abnormalities were not proved in comparison with the CC study with saline infusion.

In conclusion, the main benefit of our study is the fact that the effect of acute hyperglycemia and/or hyperinsulinemia on immune functions of PMN cells was studied in healthy subjects in vivo under conditions that may reflect diabetes-related mechanisms better than in vitro experiments. We believe that our results encourage further studies to evaluate possible mild changes in immune functions of PMN cells.

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References

[1] Roitt IM, Brostoff J, Male DK. Immunology. 4th ed. London: Gower Medical Publishing Ltd; 1996.
[2] Sawyer DW, Donowitz GR, Mandell GL. Polymorphonuclear neutrophils: an effective antimicrobial force. Rev Infect Dis 1989; 11:S1532-44.
[3] Furukawa S, Saito H, Matsuda T, et al. Relative effects of glucose and glutamin on reactive oxygen intermediate production by neutrophils. Shock 2000;13:274-8.
[4] Miller ME. Phagocytic cell functions in diabetes mellitus. In: Gupta S, editor. Immunology of clinical and experimental diabetes. New York: Plenum; 1984. p. 369-83.
[5] Johnson RB, Newman SL. Chronic granulomatous disease. Pediatr Clin North Am 1977;24:365-76.
[6] Reeves WG, Wilson RM. Infection, immunity and diabetes. In: Alberti KGMM, DeFronzo RA, Keen H, Zimmet P, editors. International textbook of diabetes mellitus. Chichester (UK): Wiley; 1992. p. 1165-71.
[7] Clark RA, Szot S. Chemotactic factor inactivation by stimulated human neutrophils mediated by myeloperoxidase-catalyzed methionine oxidation. J Immunol 1982;128:1507-13.
[8] Fialkow L, Chan CK, Grinstein S, et al. Regulation of tyrosine phosphorylation in neutrophils by the NADPH oxidase. Role of reactive oxygen intermediates. J Biol Chem 1993;268:17131-7.
[9] Murray HW, Spitalny GL, Nathan CF. Activation of mouse peritoneal macrophages in vitro and in vivo by interferon-gamma. J Immunol 1985;134:1619-22.
[10] Donadebian HD. Congenital and acquired neutrophil abnormalities. In: Klempner MS, editor. Phagocytes and disease. Dordrecht: Kluwer; 1989. p. 103-18.
[11] Robinson JP, Carter WO. Flow cytometric analysis of granulocytes. In: Bauer KD, editor. Clinical flow cytometry, principles and applications. Baltimore: Williams and Wilkins; 1993. p. 405-33.
[12] Mowat AG, Baum J. Chemotaxis of polymorphonuclear leukocytes from patients with diabetes mellitus. N Engl J Med 1971;284:621-7.
[13] Bagdade JD, Stewart M, Walters E. Impaired granulocyte adherence. A reversible defect in patients with poorly controlled diabetes mellitus. Diabetes 1978;27:677-81.

[14] Shah SV, Wallin JD, Eilen SD. Chemiluminescence and superoxide anion production by leukocytes from diabetic patients. J Clin Endocrinol Metab 1983;57:402-9.
[15] Delamaire M, Maugendre D, Moreno M, et al. Impaired leukocyte functions in diabetic patients. Diabet Med 1997;14:29-34.
[16] Vučič M, Gavella M, Božikov V, et al. Superoxidedismutase activity in lymphocytes and polymorphonuclear cells of diabetic patients. Eur J Clin Chem Clin Biochem 1997;35:517-21.
[17] Wilson RM, Tomplinson DR, Reeves WG. Neutrophil sorbitol production impairs oxidative killing in diabetes. Diabet Med 1987;4: 37-40.
[18] Walrand S, Guillet C, Boirie Y, et al. In vivo evidences that insulin regulates human polymorphonuclear neutrophil functions. J Leukoc Biol 2004;76:1104-10.
[19] Esmann V, Solling H, Juhl H. Allosteric and covalent control of glycogen synthase from leukocytes. Int J Biochem 1981;13:1123-6.
[20] Sentochnik DE, Eliopoulos GM. Infection and diabetes. In: Zahn CR, Weir GC, editors. Joslin's diabetes. 13th ed. Philadelphia: Lea and Febiger; 1994. p. 867-88.
[21] Pozzilli P, Leslie RDG. Infections and diabetes: mechanisms and prospects for prevention. Diabet Med 1994;11:935-41.
[22] Bessman AN, Sapico FL. Infections in diabetic patients: the role of immune dysfunction and pathogen virulence factors. J Diabetes Complications 1992;6:258-62.
[23] Marhoffer W, Stein M, Maeser E, et al. Impairment of polymorphonuclear leukocyte function and metabolic control of diabetes. Diabetes Care 1992;15:256-9.
[24] Sato N, Shimizu H, Suwa K, et al. MPO activity and generation of active O_2 species in leukocytes from poorly controlled diabetic patients. Diabetes Care 1992;15:1050-2.
[25] Boland OM, Blackwell CC, Clarke BF, Ewing DJ. Effects of ponalrestat, an aldose reductase inhibitor, on neutrophil killing of *Escherichia coli* and autonomic function in patients with diabetes mellitus. Diabetes 1993;42:336-40.
[26] Llorente L, De La Fuente H, Richaud-Patin Y, et al. Innate immune response mechanisms in non–insulin dependent diabetes mellitus patients assessed by flow cytoenzymology. Immunol Lett 2000;74: 239-44.
[27] Cendoroglo M, Sundaram S, Jaber BL, et al. Effect of glucose concentration, osmolality, and sterilization process of peritoneal dialysis fluids on cytokine production by peripheral blood mononuclear cells and polymorphonuclear cell functions in vitro. Am J Kidney Dis 1998;31:273-82.
[28] Lin X, Candlish JK, Thai AC. Superoxide production by neutrophils from diabetics and normal subjects in response to glucose and galactose. Exp Mol Pathol 1993;58:229-36.
[29] Tebbs SE, Lumbwe CM, Tesfaye S, et al. The influence of aldose reductase on the oxidative burst in diabetic neutrophils. Diabetes Res Clin Prac 1992;15:121-30.
[30] Thomson GA, Fisher BM, Gemmel CG, et al. Attenuated neutrophils respiratory burst following acute hypoglycemia in diabetic patients and normal subjects. Acta Diabetol 1997;34:253-6.
[31] World Health Organization Expert Committee. Definition, diagnosis and classification of diabetes mellitus and its complications. Report of WHO consultation: Part 1. Diagnosis and classification of diabetes mellitus. Geneva (Switzerland): WHO; 1999.
[32] DeFronzo RA, Tobin JD, Andres R. Glucose clamp technique: a method for quantifying insulin secretion and resistance. Am J Physiol 1979;237:214-23.
[33] Pelikánová T, Kazdová L, Chvojková Š, et al. Serum phospholipid fatty acid composition and insulin action in type 2 diabetic patients. Metabolism 2001;50:1472-8.
[34] Beatty OL, Harper R, Sheridan B, et al. Insulin resistance in offspring of hypertensive parents. BMJ 1993;307:92-6.
[35] Grzybowski J, Janiak MK, Oidak E, et al. New cytokine dressings: II. Stimulation of oxidative burst in leukocytes in vitro and reduction of viable bacteria within an infected wound. Int J Pharm 1999;184: 179-87.
[36] Shalekoff S, Tiemessen CT, Gray CM, et al. Depressed phagocytosis and oxidative burst in polymorphonuclear leukocytes from individuals with pulmonary tuberculosis with or without human immunodeficiency virus type 1 infection. Clin Diagn Lab Immunol 1998;5:41-4.
[37] Rosse W, Bunn HF. Hemolytic anemias and acute blood loss. In: Fauci AS, editor. Harrison's principles of internal medicine. USA: McGraw-Hill; 1998. p. 659-72.
[38] Liberek T, Topley N, Jorres A, et al. Peritoneal dialysis fluid inhibition of phagocyte function: effect of osmolarity and glucose concentration. J Am Soc Nephrol 1993;3:1508-15.
[39] Nielson CP, Hindson DA. Inhibition of polymorphonuclear leukocyte respiratory burst by elevated glucose concentrations in vitro. Diabetes 1989;38:1031-5.
[40] Safronova VG, Gabdoulkhakova AG, Miller AV, et al. Variations of the effect of insulin on neutrophil respiratory burst. The role of tyrosine kinases and phosphatases. Biochemistry 2001;66:840-9.
[41] Cavalot F, Anfossi G, Russo I, et al. Insulin, at physiological concentrations, enhances the polymorphonuclear leukocyte chemotactic properties. Horm Metab Res 1992;24:225-8.
[42] Esmann V. The polymorphonuclear leukocyte in diabetes mellitus. J Clin Chem Clin Biochem 1983;21:561-7.
[43] Spagnoli A, Spadoni GL, Sesti G, et al. Effect of insulin on hydrogen peroxide production by human polymorphonuclear leukocytes. Studies with monoclonal anti-insulin receptor antibodies, and an agonist and an inhibitor of protein kinase C. Horm Res 1995;43: 286-93.
[44] Hu H, Hjemdahl P, Li N. Effects of insulin on platelet and leukocyte activity in whole blood. Thromb Res 2002;107:209-15.
[45] Stites DP, Terr AI. Basic and clinical immunology. 8th ed. Stamford: Appleton and Lange; 1994.