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Discrimination between granulocytes from type I and type II diabetics patients by calorimetry

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Abstract

We report the application of calorimetric method for evaluating the metabolic response of human granulocytes either from type I-insulin-dependent diabetes mellitus (IDDM) or from type II-noninsulin-dependent diabetes mellitus (NIDDM) in comparison to cells of healthy donors. Granulocytes either from patients (type I or type II diabetics patients) or from healthy subjects were exposed in a calorimeter to 4-β-phorbol-12,13-dibutyrate (PDB) or Zymosan opsonized particle (ZC3b). The total heat released by both PDB-activated or phagocyting granulocytes from type I-IDDM patients was significantly higher than that produced by granulocytes either from type II-NIDDM patients or from healthy subjects ($p < 0.05$). The heat production by granulocyte from healthy subjects and from type II-NIDDM patients were similar ($p > 0.05$). These results demonstrate, for the first time, the discrimination between human granulocytes from type I-IDDM and type II-NIDDM patients by calorimetry. Thus, we suggest that due to its high sensitivity, calorimetry may be used as an auxiliary method for discriminating type I-IDDM from type II-NIDDM patients based on granulocyte reactivity.

Keywords: Calorimetric assay; Diabetes; Granulocytes; Phagocytosis; Phorbol ester (PDB)

1. Introduction

Calorimetry has been applied to studies of microbial and Leishmania metabolism [1,2], metabolic activity of red cells, granulocytes, platelets and lymphocytes [3], cancer research [4], phagocytosis [5,6], and antigen–antibody reaction [7,8]. Eftimiadi and Rialdi [9] reported the correlation between oxygen consumption and release of heat in human neutrophils under activation with PMA. Other studies have demonstrated that calorimetry is a highly sensitive technique for assessing cellular function and for estimating the metabolic rate of human blood cells. Kemp et al. [10] have reported the increase of heat production in macrophage hybridoma stimulated with PMA. Ljunggren et al. [11] demonstrated the effect of cellular interaction (granulocytes and erythrocytes) on metabolic pathways by calorimetry. Significant alterations in the heat production by blood cells under several conditions have been reported [12–16]. In a previous report, we demonstrated that human peripheral blood mononuclear cells from non-

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infected or from chronically *S. mansoni* infected patients can be discriminated based on their reactivities by microcalorimetry [17,18].

Thus, we have hypothesized that cells of patients may have altered reactivity when compared with those of normal individuals. Based on this idea, we have tested comparatively the reactivity of granulocytes from diabetic patients type I-insulin-dependent diabetes mellitus (IDDM) and type II-non-insulin-dependent diabetes mellitus (NIDDM) in comparison to cells from healthy subjects. In the present study, we evaluate the calorimetric granulocyte response during phagocytosis of opsonized particles or under stimulation with phorbol ester (an activator of protein kinase C). The discrimination between granulocytes from type I-IDDM and type II-NIDDM based on heat production was clearly demonstrated.

2. Experimental

2.1. Diabetic patients

The ethical committee of Santa Casa Hospital of Belo Horizonte, Brazil, approved this study and the consent of all the participants was obtained. All volunteers were subjected to detailed physical examination and evaluation of medical history and laboratory data before the study. Fifty-two non-diabetics controls, 50 type II-NIDDM diabetics patients with age ranging from 45 to 70 years, and 53 type I-IDDM patients were first admitted for investigation. Twenty-two people from non-diabetic controls, 20 type II diabetic patients and 23 type I-NIDDM patients were excluded based on the exclusion criteria by presenting one or more of the conditions or pathology listed: smokers, hypertension treatment, cardiac insufficiency, pregnancy, alcoholism, dementia, inflammation, malignant disease, infection, or fasting plasma glucose >110 but <140 mg/dl. The healthy non-diabetic controls were 30 people and they were selected by Hemominas Foundation and by Dr. Ataualpa Pereira Reis (CLIMED). Both types I and II patients were formed by 30 people and they were selected by Dr. Victor Pardini and by Dr. Francisco Chagas Lima e Silva at the Santa Casa Hospital of Belo Horizonte and at the General Clinic Service of Clinics Hospital, Federal University of Minas Gerais (UFMG). The inclusion criteria for type I-IDDM patients was cutoff of glucose >140 mg/dl; age ranging from 20 to 70 years, and all of them were taking insulin. For type II-NIDDM patients, plasmatic glucose was also considered, the age ranging from 40 to 70 years, and requiring non-insulin.

2.2. Cell separation

Human granulocytes were purified from 10.0 ml heparinized venous blood. Suspensions containing granulocytes were prepared according to the method described by Bicalho et al. [19]. The cells were counted in a Neubauer chamber and the viability of each sample was greater than 90% as determined by the Trypan Blue exclusion test. The granulocyte suspension used in the experiments always showed a percentage lesser than 10 for eosinophils and greater than 90 for neutrophils without contamination with mononuclear cells (monocytes and lymphocytes) and erythrocytes.

2.3. Calorimetric assay

Calorimetry was performed as described by Chaves et al. [17] in a heat conduction batch-mixing calorimeter similar to Wadso's [20], built in our laboratory and in USA, according to Lovrien et al. [21].

The Seebeck thermopiles have a conversion factor (figure of merit) of 7 $\mu W\ \mu^{-1}$ and it has been indicated by the manufacturer. The calorimeter consists of two reaction vessels or cells, sandwiched between thermopiles and enclosed in an aluminum block that acts as a heat sink. The calorimeter mixing vessels (two) made of gold has a small and a large compartment holding up to 1.0 and 0.5 ml, respectively, in a total volume of 5.0 ml. In a typical test, 1×10^6 granulocytes either from non-diabetic subjects, from type I-IDDM or from type II-NIDDM diabetics patients in 1.0 ml were added to the large compartment, and either 50 μl of Zymosan opsonized particles (13 mg/ml) or of PDB were added to the smaller compartment. In all experiments, cells and reagents were diluted with minimum essential medium Eagle (MEM) and each compartment contained a final volume of 1.0 ml. In the control vessel, granulocytes were added to the large compartment and MEM were added to the smaller one. Mixing of the vessel for aeration was done at 5 or 10 min intervals, according to the need, but this was constant for each type of experiment. Thermal equilibrium in

the cells was reached after 10–25 min of pre-incubation. Amplifier gains of ×1000 were used. Calibration was performed by neutralization of Tris by HCl based on $\Delta H^0 = -47\,267$ J/mol of H^+ neutralized. Standard calibration plots were described by $A = -92.52 + 299.46q$, where A is the integrated area under the output envelope and q the calculated amount of heat released. All heat production values were exothermic quantities, and they were registered in an electronic recorder coupled to the microcalorimeter. After each calorimetric assay, cell viability was evaluated by the Trypan Blue exclusion test.

2.4. Statistical analysis

The statistical analysis was performed using the unpaired Student t-test using the software Microcal Origin 3.0. A $p < 0.05$ was taken as the threshold of significance.

3. Results and discussion

Diabetes mellitus is considered to be a multifactorial disease. The type I-IDDM involves damage of the pancreatic beta cells and blockade of insulin secretion, while type II-NIDDM depends on the progressive development of insulin resistance, possibly related to a decrease in the insulin-receptor tyrosine kinase [22,23].

The microcalorimetric assay now presented for assessing the metabolic response of granulocytes from diseased patients (type I and II diabetics patients) and from non-diseased subjects is based on the determination of the heat production using a calorimeter.

Our present results represent the metabolic response of granulocytes during phagocytosis of opsonized particles or under stimulation with phorbol ester (an activator of protein kinase C) measured by calorimetry. The results were expressed as total heat production in a given time interval in mJ over 1 h or as the heat production rate in pW per cell.

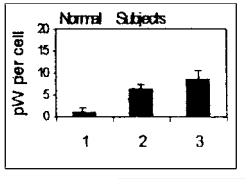
The metabolic response of human Granulocytes from diabetic patients (type I and II) stimulated with PDB or during phagocytosis are shown in Table 1 and Fig. 1. In Table 1 (line 2) the results with phagocyting granulocytes are shown. Cells from type I-IDDM patients had greater heat production than those from non-diabetic subjects and from type II-NIDDM patients ($p < 0.05$). The direct comparison between the heat produced by granulocytes from type II-NIDDM and non-diabetic subjects was not significant ($p > 0.05$). These results clearly demonstrate, for the first time, that granulocytes from type I-IDDM patients can be discriminated from non-diabetic subjects and from type II-NIDDM patients by calorimetry. The phagocytosis of opsonized particles by human granulocytes involves the activation of several biochemical pathways. It has been reported that during phagocytosis there is activation of phosphatidylinositol cascade [24], as well as O_2 consumed, inositoltriphosphate (IP3) and diacylglycerol production [25]. The diacylglycerol is the physiological activator of protein kinase C (PKC). The results in which the effect of phorbol ester (PDB), an activator of PKC, was evaluated by calorimetry appears in Table 1 (line 3). These results were similar to those shown in Table 1 (line 2) with phagocyting granulocytes. The stimulation of PKC with PDB was also able to discriminate the granulocytes from type I-IDDM in comparison to cells from non-diabetic subjects and from type II-NIDDM patients (Table 1). The total heat produced and the heat production rate were both significantly ($p < 0.05$) increased in granulocytes from type I-IDDM patients.

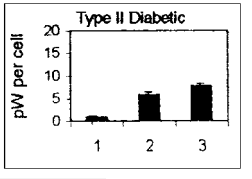
Stimulation of granulocytes function with PDB has been accepted as an important step in cell activation [26]. The alteration in PKC translocation from cytosol to membrane or altered reactivity to PDB stimulation in granulocytes from type I-IDDM patients may be suggested as the explanation for the difference found. These ‘‘in vitro’’ altered metabolic response detected in granulocytes from type I-IDDM patients during a phagocytic process and under stimulation with PDB may be reflexes of an adapted metabolic response which is disease-induced. The shapes of the curves were distinct when phagocyting or PDB-stimulated granulocytes from type I-IDDM (Fig. 1, panels B and E) were compared to the cells from type II-NIDDM patients (Fig. 1, panels C and F) and from non-diseased subjects (Fig. 1, panels A and D).

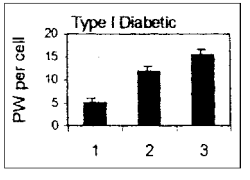
It is accepted that granulocytes are involved in the inflammatory process that leads to tissue injury. It is also known that the activation of granulocytes leads to migration, phagocytosis, degranulation, production of oxidizing species, and phospholipid turnover [27,28]. These factors, in conjunction with our present results on granulocytes functions, may suggest that cells from

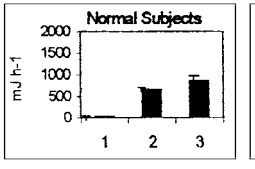
Table 1
Heat released by human granulocytes (G) from diabetics patients (types I and II) and from non-diseased subjects under stimulation with opsonized particles (ZC3b) or phorbol ester (PDB)

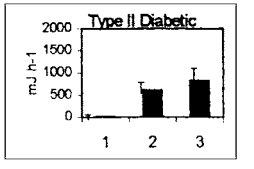
Experiments	Heat production rate ± S.D. (PW per cell)[a]			Total released heat ± S.D. (mJ h^{-1})[b]		
	Normal subjects	Type I diabetic	Type II diabetic	Normal subjects	Type I diabetic	Type II diabetic
(1) G + medium	1.0 ± 0.05	5.1 ± 0.3	0.9 ± 0.08	14.6 ± 0.7	63.5 ± 8.3	11.0 ± 0.5
(2) G + ZC3b	6.4 ± 0.6	11.9 ± 1.6*	5.9 ± 0.5	665 ± 69.2	1.516 ± 119.3*	616 ± 59.4
(3) G + PDB	8.6 ± 1.4	15.6 ± 1.2*	7.9 ± 0.4	854 ± 75.3	1.921 ± 123.6*	836 ± 21.6

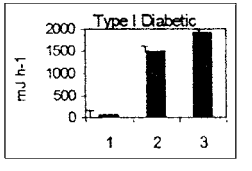












The values represent the average of 30 experiments. The panels are graphic representations of the numeric data.

[a] The steady state heat production rate was measured at the maximum peak of the power–time curve at zero slope.

[b] The total heat released was measured after 3600 s.

* Significant at $p < 0.05$ by the Student t-test when compared to the control (line 1).

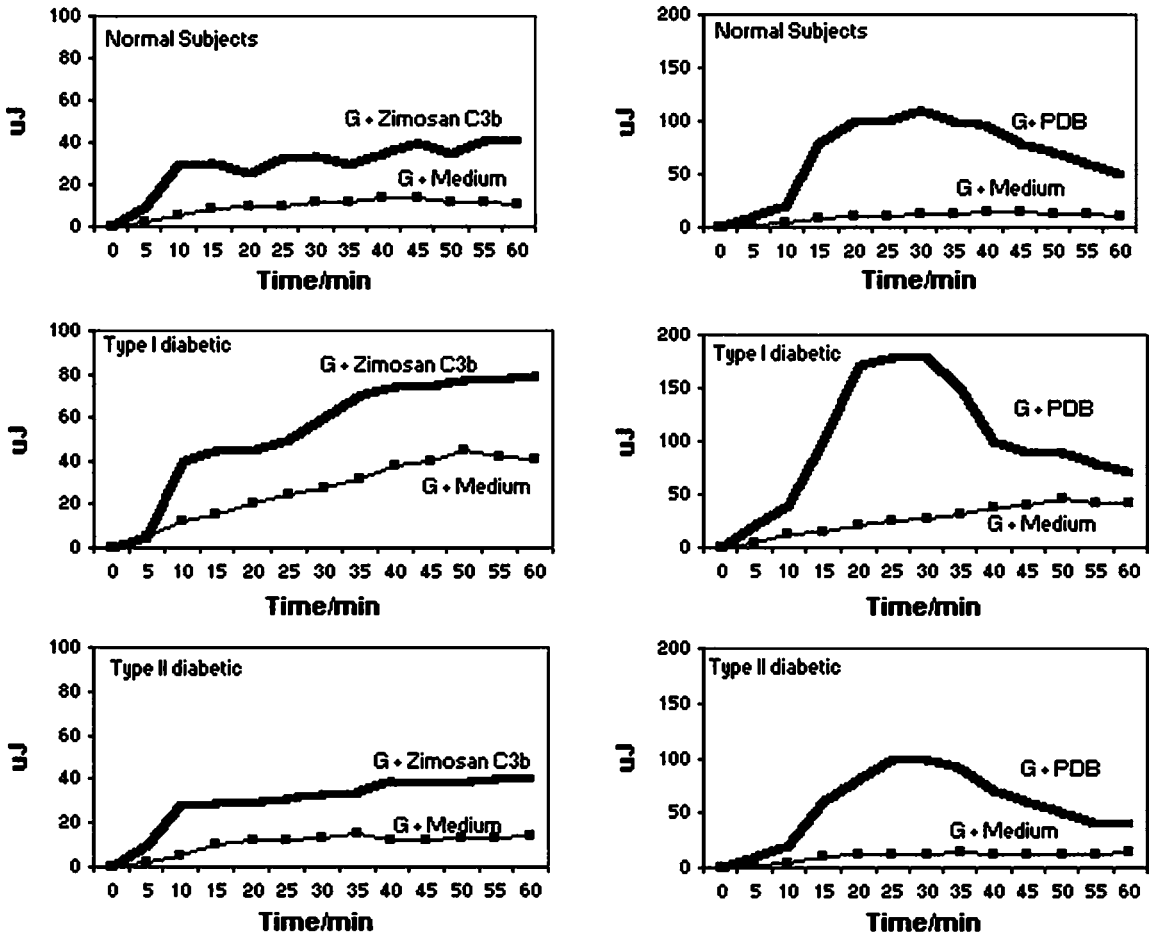


Fig. 1. Representation of typical curves for granulocytes (G) from non-diabetic subjects, from type I-IDDM patients and from type II-NIDDM patients stimulated with opsonized particles (Zymosan C3b) or with phorbol ester (PDB).

type I-IDDM patients are able to cause greater tissue damage than cells from non-diabetics subjects or from type II-NIDDM patients.

Thus, calorimetry was used to successfully discriminate granulocytes from type I and type II diabetic patients and it may have value as an auxiliary diagnostic tool in diabetes.

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