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Comparative evaluation of metabolic response of granulocytes from Type 2 diabetic patients by calorimetry and by luminol-dependent chemiluminescence assays

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Abstract

We report comparative studies on metabolic response by granulocytes from healthy subjects and Type 2 diabetic patients (T2-DM) using calorimetry and luminol-dependent chemiluminescence. Three groups of substances were used for activating or inhibiting the granulocyte reactivity: (i) opsonized zimosan particles (ZC3b), phorbol ester (PDB); (ii) cytokines (IFN-γ, GM-CSF and IL-10) and (iii) cyclic nucleotide-elevating agents (cAMP and cGMP). Results were expressed as total heat production in 1 h for calorimetric assay and relative light unit (RLU) during 60 min reaction for luminol-dependent chemiluminescence.

The results obtained with ZC3b, PDB and IFN-γ/GM-CSF showed that the metabolic response of stimulated-granulocytes were notably larger than those observed with non-stimulated cells in both techniques. The cellular metabolic response from healthy subjects and from T2-DM patients was similar. In contrast, interleukin 10 has no effect on granulocytes from T2-DM patients, but was strongly inhibitory for granulocytes from healthy subjects. The experiments with cyclic nucleotides showed an inverse metabolic response. Dibutyryl cyclic AMP inhibited both heat release and reactive oxygen species generation (ROS) in granulocytes from healthy subjects, but activated cells from T2-DM patients. 8-Br-cyclic GMP activated cells from healthy subjects and inhibited granulocytes from T2-DM patients.

Calorimetry appears to be the best technique for measuring metabolic response in stimulated-granulocytes while only the chemiluminescence assay was able to discriminate non-stimulated-granulocytes from healthy subjects and T2-DM patients. Both techniques are highly sensitive methods which could become important tools for studying signaling mechanisms in immune system cells.

Keywords: Diabetes; Calorimetric assay; Chemiluminescence assay; Cyclic nucleotides; Cytokines

1. Introduction

Diabetes mellitus produces a state of chronic hyperglycemia, which in turn leads to the development of severe complications including retinopathy, nephropathy, neuropathy, and atherosclerosis. Several links relate mitochondrial metabolism in T2-DM patients (Type 2 diabetes) to chronic hyperglycemia [1]. Among them, ATP synthesis by oxidative phosphorylation and cellular energy metabolism (ATP/ADP ratio), redox status and reactive oxygen species (ROS) production, membrane potential and substrate transport across the mitochondrial membrane. Recent findings, such as (a) mitochondrial ROS production is central in the signaling pathway of harmful effects of hyperglycemia; (b) AMPK activation is a major regulator of both glucose and lipid metabolism connected with cellular energy status; (c) hyperglycemia by

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inhibiting glucose-6-phosphate dehydrogenase (G6PDH) by a cAMP mechanism plays a crucial role in NADPH/NADP ratio and thus in the pro-oxidant/anti-oxidant cellular status, have deeply changed the view of diabetes and related complications [2].

Studies on ROS generation and/or heat release in ageing, human schistosomiasis and diabetes have been reported [3–8]. Both are highly sensitive techniques able to discriminate between diseased and non-diseased patients. The physicochemical approach for testing cellular reactivity at energy metabolism and at cellular redox status may be useful for understanding the mechanisms involved in the pathogenesis of diabetes.

Recently, we have demonstrated that cyclic AMP-elevating agents induce an inverse metabolic response for ROS and NO generation by granulocytes from T2-DM patients [9,10]. These results suggest a disease-induced metabolic adaptation with a possible alteration in energetic metabolism.

The evaluation of the cellular reactivity could become an important tool for identification of disease-induced metabolic adaptations. The present paper compares microcalorimetric and chemiluminescence assays as methods for assessing the cellular metabolic status. Both techniques are highly sensitive methods that have been previously applied to studies of metabolism of blood cells from healthy and from patients with different pathological conditions [11–17]. Thus, our main intention in the present paper was to compare the energetic and oxidative metabolism in granulocytes from T2-DM patients with granulocytes from healthy subjects.

2. Experimental

2.1. Reagents

Dibutyryl cyclic AMP, 8-Br-cyclic GMP and PDB (phorbol 12,13-dibutyrate) were purchased from Sigma Co., Interleukin 10 (IL-10) (DNAX), GMC-SF was used as supernatant of cell culture (RJCB collection—CR070-cell line 5637) and IFN-γ was supernatant of mononuclear cells (1×10^6 mL) primed with Con A (7.5 μg) and cultured 24 h at 37 °C in a humidified CO_2 incubator.

2.2. Granulocyte separation

Granulocytes were purified from 10.0 ml of heparinized venous blood using the Ficoll-Hypaque gradient according to Bicalho et al. [18] with slight modifications. The cellular viability of each sample was always greater than 95% as determined by the Trypan blue exclusion test.

2.3. Diabetic patients

The Ethical Committee from Santa Casa Hospital of Belo Horizonte, Brazil approved this study and informed consent was obtained of all participants. All volunteers were submitted to detailed physical examination, evaluation of medical history and laboratory data, before entering the study. Volunteers presenting one or more of the conditions or pathologies were excluded: smokers, pregnancy, alcoholism, dementia, inflammation, malignant disease, infection or fasting plasma glucose >110 to <140 mg/dl. The healthy subjects were 28 people (15 women and 13 men) and T2-DM patients were 20 people (11 women and 9 men) and were selected by Dr. Maria Regina Calsolari at the Santa Casa Hospital of Belo Horizonte and Dr. Francisco das Chagas Lima e Silva at Hospital das Clínicas of Federal University of Minas Gerais (UFMG). The inclusion criteria were age range (45–70 years old) cutoff for fasting plasma glucose >140 mg/dl, non-insulin requiring and taking pills of clorpropamide. Mean age of controls was 56.4 ± 11.0 (S.D.) years and of diabetics, 56.8 ± 13.0 years.

2.4. Luminol-dependent chemiluminescence assay—ROS quantification

The quantitative ROS determination was performed in a luminol-dependent chemiluminescence assay by the incubation of granulocytes (1×10^6 cells/100 μl Hank's balanced salt solution, HBSS) either with 100 μl of cyclic AMP (dibutyryl cyclic AMP) (10^{-4} M); 100 μl of cyclic GMP (8-Br-cyclic GMP) (10^{-4} M), 10 μl of PDB (10^{-4} M), 100 μl of IFN-γ and GMC-SF, 10 μg/5 μl of interleukin 10 (IL-10) or with 50 μl of opsonized zimosan particles (13 mg/ml). A luminol [Sigma Co.] stock solution was made by dissolving 1.77 mg of luminol in 1.0 ml dimethyl sulphoxide (DMSO) to give a concentration of 10^{-2} M. Before use this was diluted further to 10^{-4} M in PBS (pH 7.3). The chemiluminescence measurements were performed in a luminometer (LUMAT, LB 9501, EG&G BERTHOLD, Germany). The experiments were done in duplicate and carried out at 37 °C. The chemiluminescence was recorded during 60 min, time enough for observation of the peak. The results were expressed in RLU/60 min (relative light unit per hour), as total chemiluminescence produced. The control experiments were done simultaneously. The concentration of 10^{-4} M used for experiments with cyclic AMP and GMP-elevating agents and PDB were based on a dose–response curve in which the following concentrations of reagents were tested: 10^{-2}, 10^{-4}, 10^{-6} and 10^{-8} M. The concentration (10^{-4} M) corresponds to a higher activation or inhibition of ROS production in conjunction with a lower percentage of dead cells (<5%).

2.5. Calorimetric assay

Calorimetry was performed as described by Chaves et al. [19] in a heat conduction batch-mixing calorimeter similar to Wadso's [20], built in our laboratory and in the USA according Lovrien et al. [21].

The Seebeck thermopiles have a conversion factor (Figure of merit) of 7 μW. The calorimeter consists of two reaction vessels, sandwiched between thermopiles and enclosed in an

aluminum block that act as a heat sink. The calorimeter mixing vessels, made of gold, have two compartments holding up to 1.0 and 0.5 ml, in a total volume of 5.0 ml. In a typical test, 1×10^6 granulocytes either from healthy subjects or from T2-DM patients in 1.0 ml were added to the large compartment and either 100 μl of cyclic AMP (dibutyryl cyclic AMP) (10^{-4} M); 100 μl of cyclic GMP (8-Br-cyclic GMP) (10^{-4} M), 10 μl of PDB (10^{-4} M), 100 μl of IFN-γ and GMC-SF, 10 μg/5 μl of interleukin 10 (IL-10) or 50 μl of opsonized zimosan particles (13 mg/ml) were added to the smaller compartment. In all experiments, cells and reagents were diluted with RPMI (Culture Medium) and each vessel contained a final volume of 1.0 ml. In the control vessel, granulocytes were added to the large compartment and RPMI 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 preincubation. Amplifier gains of 1000× were used. Calibration was performed by neutralization of Tris by HCl based on $\Delta H^\circ = -47\,267$ 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 were registered in an electronic recorder coupled to the microcalorimeter. Calorimetric assay results were expressed as total heat production in 1 h. After each calorimetric assay, cell viability was evaluated by the Trypan blue exclusion test.

2.6. Statistical analysis

The statistical analysis was performed using the unpaired Student's *t*-test using the software Microcal Origin 5.0. A $P < 0.05$ was taken as the threshold of significance.

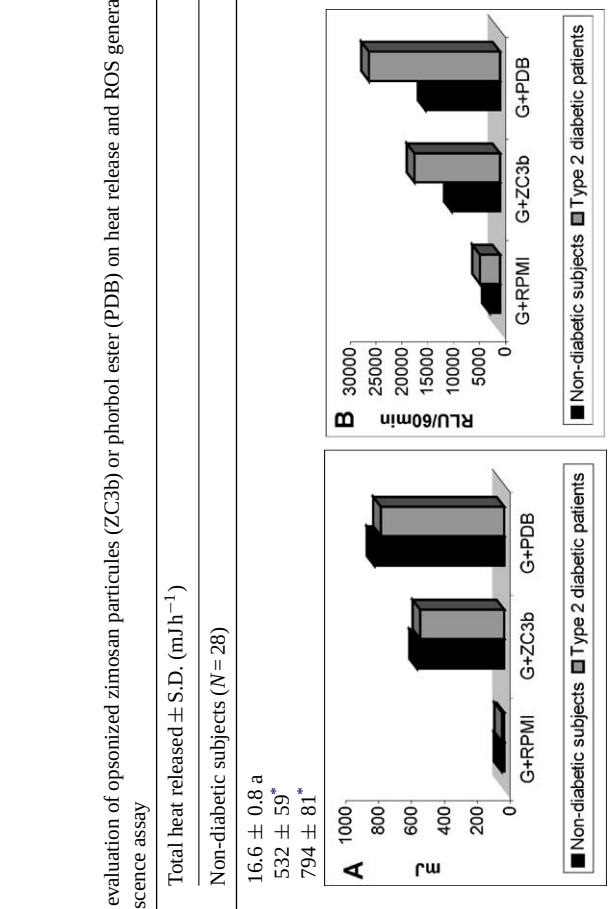
3. Results and discussion

Both techniques are able to assess the metabolic function of granulocytes from healthy subjects and from T2-DM patients. The calorimetric assay appears to be more efficient for studying stimulated-granulocytes while only the chemiluminescence assay is able to discriminate the metabolic basal response in granulocytes from healthy subjects when compared to granulocytes from T2-DM patients.

Granulocytes either from healthy subjects or from T2-DM patients were activated, inhibited by several kinds of substances. The results are shown in Tables 1–3. The metabolic response in stimulated-granulocytes was notably larger than that with non-stimulated cells from the controls (Tables 1 and 2, line 1—Panels A and B). This increased metabolic response was observed for granulocytes from healthy and from T2-DM patients. On the other hand, while, IL-10 was strongly inhibitory for granulocytes from healthy subjects no effect was observed on granulocytes from

Table 1
Comparative evaluation of opsonized zimosan particules (ZC3b) or phorbol ester (PDB) on heat release and ROS generation by granulocytes from healthy subjects and from T2-DM patients by calorimetry and chemiluminescence assay

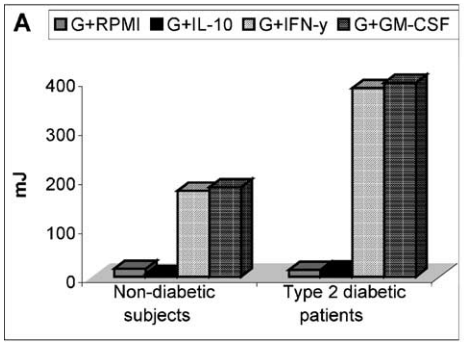
Experiments	Total heat released ± S.D. (mJ h^{-1})		Total ROS generation (RLU/60 min ± S.D.)	
	Non-diabetic subjects ($N = 28$)	T2-DM patients ($N = 25$)	Non-diabetic subjects ($N = 28$)	T2-DM patients ($N = 25$)
1. G + RPMI	16.6 ± 0.8 a	14.4 ± 0.9 b	1932 ± 182 c	3842 ± 294 d
2. G + ZC3b	532 ± 59*	513 ± 53*	9342 ± 732*	16433 ± 153*
3. G + PDB	794 ± 81*	752 ± 69*	14392 ± 132*	25343 ± 232*

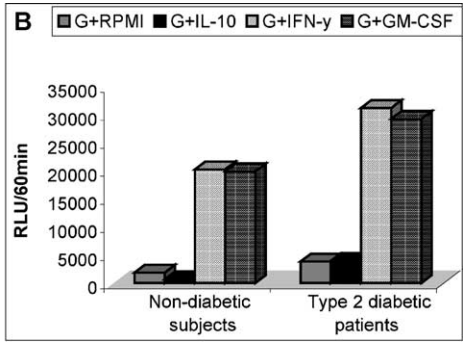


T2-DM: Type 2 diabetes mellitus. *N*: number of experiments. Total heat released was expressed as milliJoule (mJ) during 60 min reaction. Reactive oxygen species (ROS) generation was expressed as relative light units (RLU) during 60 min reaction. G: granulocytes; ZC3b: opsonized zimosan particle; PDB: phorbol ester; letter c differ from letter d at $P < 0.05$ by Student's *t*-test; letter a was not significant when compared to letter b ($P < 0.05$).
* Significant at $P < 0.05$ by Student's *t*-test when compared to the respective control (line 1).

Table 2
Calorimetry and chemiluminescence assays: comparative evaluation of the effect of cytokines on reactivity of granulocytes from T2-DM patients and from healthy subjects

Experiments	Total heat released ± S.D. (mJ h^{-1})		Total ROS generation (RLU/60 min ± S.D.)	
	Non-diabetic subjects ($N = 28$)	T2-DM patients ($N = 25$)	Non-diabetic subjects ($N = 28$)	T2-DM patients ($N = 25$)
1. G + RPMI	15.9 ± 0.5 a	13.5 ± 0.5 b	1849 ± 174 c	3795 ± 301 d
2. G + IFN-y	175 ± 2.9*	384 ± 2.7*	20184 ± 195*	31142 ± 284*
3. G + GM-CSF	182 ± 3.2*	395 ± 3.5*	19854 ± 172*	29184 ± 232*
4. G + IL-10	5.3 ± 0.6*	13.9 ± 0.4*	634 ± 53*	3932 ± 304*



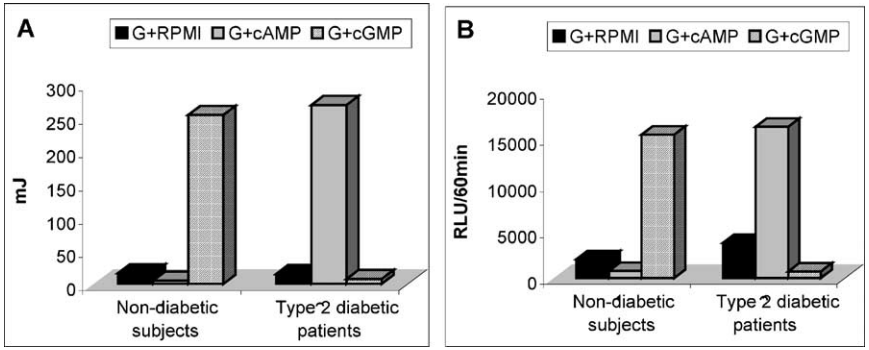


T2-DM: Type 2 diabetes mellitus. *N*: number of experiments. Total heat released was expressed as milliJoule (mJ) during 60 min reaction. Reactive oxygen species (ROS) generation was expressed as relative light units (RLU) during 60 min reaction. G: granulocytes; IL-10: Interleukin 10; IFN-γ: interferon gamma and GM-CSF: factor stimulator colony of macrophage and granulocytes; letter c differ from letter d at $P < 0.05$ by Student's *t*-test; letter a was not significant when compared to letter b ($P < 0.05$).

* Significant at $P < 0.05$ by Student's *t*-test when compared to the respective control (line 1).

Table 3
Comparative evaluation of cyclic nucleotides on heat release and ROS generation by granulocytes from T2-DM patients and from healthy subjects by calorimetry and chemiluminescence assays

Experiments	Total heat released ± S.D. (mJ h^{-1})		Total ROS generation (RLU/60 min ± S.D.)	
	Non-diabetic subjects ($N = 28$)	T2-DM patients ($N = 25$)	Non-diabetic subjects ($N = 28$)	T2-DM patients ($N = 25$)
1. G + RPMI	16.1 ± 0.7 a	15.1 ± 0.9 b	2036 ± 194 c	3784 ± 325 d
2. G + cAMP	5.0 ± 0.3*	269 ± 2.5*	748 ± 63*	16324 ± 1592*
3. G + cGMP	254 ± 22*	7.4 ± 0.6*	15482 ± 1483*	85 ± 72*



T2-DM: Type 2 diabetes mellitus. *N*: number of experiments. Total heat released was expressed as milliJoule (mJ) during 60 min reaction. Reactive oxygen species (ROS) generation was expressed as relative light units (RLU) during 60 min reaction. G: granulocytes; cAMP: cyclic dibutyryl AMP and cGMP: cyclic 8-Br GMP; letter c differ from letter d at $P < 0.05$ by Student's *t*-test; letter a was not significant when compared to letter b ($P < 0.05$).

* Significant at $P < 0.05$ by Student's *t*-test when compared to the respective control (line 1).

diabetic patients (Table 2, line 4—Panels A and B), suggesting that the IL-10 signaling pathway in cells with adapted metabolism differ from those from healthy subjects. The absence of cellular reactivity needs further study.

Our results clearly demonstrated that the calorimetric assay was a better technique for studying the metabolic response in stimulated-granulocytes than the luminol-dependent chemiluminescence test. The results with calorimetry expressed as the ratio between granulocytes in the presence (E) or in the absence (C) of ZC3b or PDB were, respectively: 32.0; 47.8 for healthy subjects and 35.6; 52.2 for T2-DM patients. In a chemiluminescence assay the E/C ratio were 4.8 (ZC3b); 7.4 (PDB) for healthy subjects and 4.2 and 6.5 for, respectively, for T2-DM patients (Table 1—Panels A and B). Similar results were observed with cytokine (Table 2) and cyclic nucleotides (Table 3). The chemiluminescence assay seems to be better than calorimetry for studying metabolic basal response in non-stimulated-granulocytes.

The basal metabolic response of granulocytes from healthy subjects and from T2-DM patients, were clearly discriminated by the chemiluminescence assay, but not by calorimetry (Tables 1–3, line 1—Panels A and B).

We have previously observed that peripheral blood mononuclear cells from *S. mansoni* infected patients showed a delayed calorimetric burst and decreased heat production rate [8], alteration in the cytotoxicity, blastogenesis and in the inositolpolyphosphate (IPx) contents [22,23]. Based on present results in conjunctions with others [6] we have suggested that cells from ill patients and from healthy subjects may have different reactivity under metabolic stimulation as a consequence of a disease-induced metabolic adaptation.

Dibutyryl cyclic AMP inhibited both the heat release and ROS generation by granulocytes from heat subjects, but activated in cells from T2-DM patients (Table 3, line 2—Panels A and B). By contrast, 8-Br-cyclic GMP activated cells from non-diabetic and inhibited granulocytes from T2-DM patients (Table 3, line 3—Panels A and B). We have previously reported similar results with nitric oxide (NO) generation and ROS production in diabetes [9,10]. Both methods used in present paper were able to detect this inverse metabolic response (Table 3—Panels A and B) and our present results reinforce our hypothesis on disease-induced metabolic adaptation and suggest that both calorimetric and chemiluminescence assays are reliable techniques for studying signalling mechanisms and of discrimination of the cellular reactivity in cells from healthy subjects in comparison to cells from ill patients.

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