

# Correlation Between ROS Production and InsP<sub>3</sub> Released by Granulocytes from Type 1 Diabetic Patients in a cAMP-Dependent Manner

Míriam Martins Chaves<sup>\*1</sup>, Daniela Caldeira Costa<sup>1</sup>, Denniece Adriana da Costa Souza<sup>2</sup>, Francisco das Chagas Lima e Silva<sup>2</sup> and José Augusto Nogueira Machado<sup>2</sup>

<sup>1</sup>Departamento de Bioquímica/Imunologia - ICB, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil

<sup>2</sup>Hospital Santa Casa de Belo Horizonte, Núcleo de Pesquisa e Pós-Graduação (NPPG), Belo Horizonte, MG, Brazil

**Abstract: Background:** Diabetes is associated with a pro-inflammatory status characterized by an increased production of inflammatory molecules. Reactive Oxygen Species (ROS) and cAMP elevating agents represent two molecular systems, normally generated during inflammation. These molecules could be responsible for the alteration of signaling pathways. In the present paper we have studied the correlation between ROS generation and inositolpolyphosphates (InsP<sub>1</sub>, InsP<sub>2</sub>, InsP<sub>3</sub> and InsP<sub>4</sub>) released by granulocytes from Type 1 diabetic patients (DM1) in the presence or in the absence of cyclic AMP-elevating agents.

**Methods:** The effect of cAMP on ROS production was quantified in a chemoluminescence assay luminol-dependent (RLU/min). InsP<sub>1</sub>, InsP<sub>2</sub>, InsP<sub>3</sub> and InsP<sub>4</sub> were quantified by inositol-H<sup>3</sup> in a Beta-counter and the results were expressed as count per minute (CPM).

**Results:** The elevation of intracellular level of cAMP inhibited both InsP<sub>3</sub> and ROS production in granulocytes from healthy subjects and activated in the cells from Type 1 diabetic patients. InsP<sub>1</sub>, InsP<sub>2</sub> and InsP<sub>4</sub> did not show significant alteration in both studied cells. There was a significant correlation between InsP<sub>3</sub> and ROS in the presence of elevated content of cAMP. This correlation was observed in a 15 minutes reaction for healthy subjects and in 120 minutes for DM1.

**Conclusions:** The importance of both InsP<sub>3</sub> release and ROS production in an inflammatory process and tissue pathophysiology in Type 1 diabetic patients is still under debate because hyperglycemia accelerates generation of oxidative stress and may play an important role in the development of complications in diabetes. Thus, our results demonstrated alteration in metabolic response in granulocytes from Type 1 diabetic patients and it may be important for the development of therapeutic processes and drugs that interfere with signaling of ROS generation and may contribute to the improvement of the severe complications of diabetes.

**Keywords:** Type 1 diabetes, neutrophil, InsP<sub>3</sub>, ROS.

## INTRODUCTION

It is well accepted that the human granulocytes and cytokines play an important role in host defense and inflammation [1]. The high level of Reactive Oxygen Species (ROS) produced by granulocytes appears to be involved in several pathologies including diabetes mellitus [2,3]. It has also been suggested that the hyperglycemia of diabetes may induce several adaptations in metabolic signaling pathways [4]. Some of these adaptations implicate an altered metabolic response. We have previously demonstrated that the intracellular elevation of cAMP activates ROS and NO production by granulocytes from diabetic patients and inhibited in healthy subjects [5,6]. This inverse metabolic response might be a consequence of hyperglycemia. In this context, it has suggested that hyperglycemia activates phospholipase A2 (PLA2) and the diacylglycerol (DAG)-protein kinase C (PKC) pathways, which enhance prostanoids synthesis, such as prostaglandin E2 (PGE2) and PGI beside ROS [7-9]. PGE2 induces intracellular elevation by the activation of beta adrenergic receptor [10]. Thus, a possible association

among PGE2, cAMP and ROS can be envisaged. We have previously suggested that increased ROS production in granulocytes from Type 2 diabetic patients (DM2) involves a sequential activation of the Epac/PKB/PI-3K signaling pathway while in granulocytes from healthy subjects, the cAMP/PKA appears to be the main metabolic route [11,12]. These signaling pathways are calcium dependent, but there are few reports in the literature demonstrating this association among ROS production, inositol turnover and intracellular content of cyclic AMP. Thus, in the present paper we have studied the correlation between ROS production and inositolpolyphosphates release in the presence or in the absence of intracellular elevation of cyclic AMP in granulocytes from Type 1 diabetic patients and from healthy subjects.

## SUBJECTS AND METHODS

### Subjects

Details of the study were presented and approved by the Ethical Committee of the Santa Casa Hospital of Belo Horizonte and an appropriate informed consent was obtained from all participants. Each volunteer was subjected to a detailed physical examination, as well as to an evaluation of medical history and laboratory data, before being enrolled to the study. Type 1 diabetic patients and the healthy subjects

\*Address correspondence to this author at the Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais - Caixa Postal 486, 30161-970, Belo Horizonte, Minas Gerais, Brazil; Tel: 55 31 3409 2660; Fax: 55 31 3409 2614; E-mail: chavesmm@icb.ufmg.br

were selected by Dr. Maria Regina Calsolari (endocrinologist) and by Dr. Francisco Chagas Lima e Silva (General Clinic) at the Santa Casa Hospital of Belo Horizonte. All the patients were taking insulin; years old =  $38,0 \pm 10$ ; blood glucose =  $188 \pm 10.3$  mg/dL;  $Hb1_{AC} = 9.7 \pm 2.8$ . The control group was  $39 \pm 12$  years old; blood glucose =  $83 \pm 9.1$  and  $Hb1_{AC} = 5.5 \pm 0.4$ .

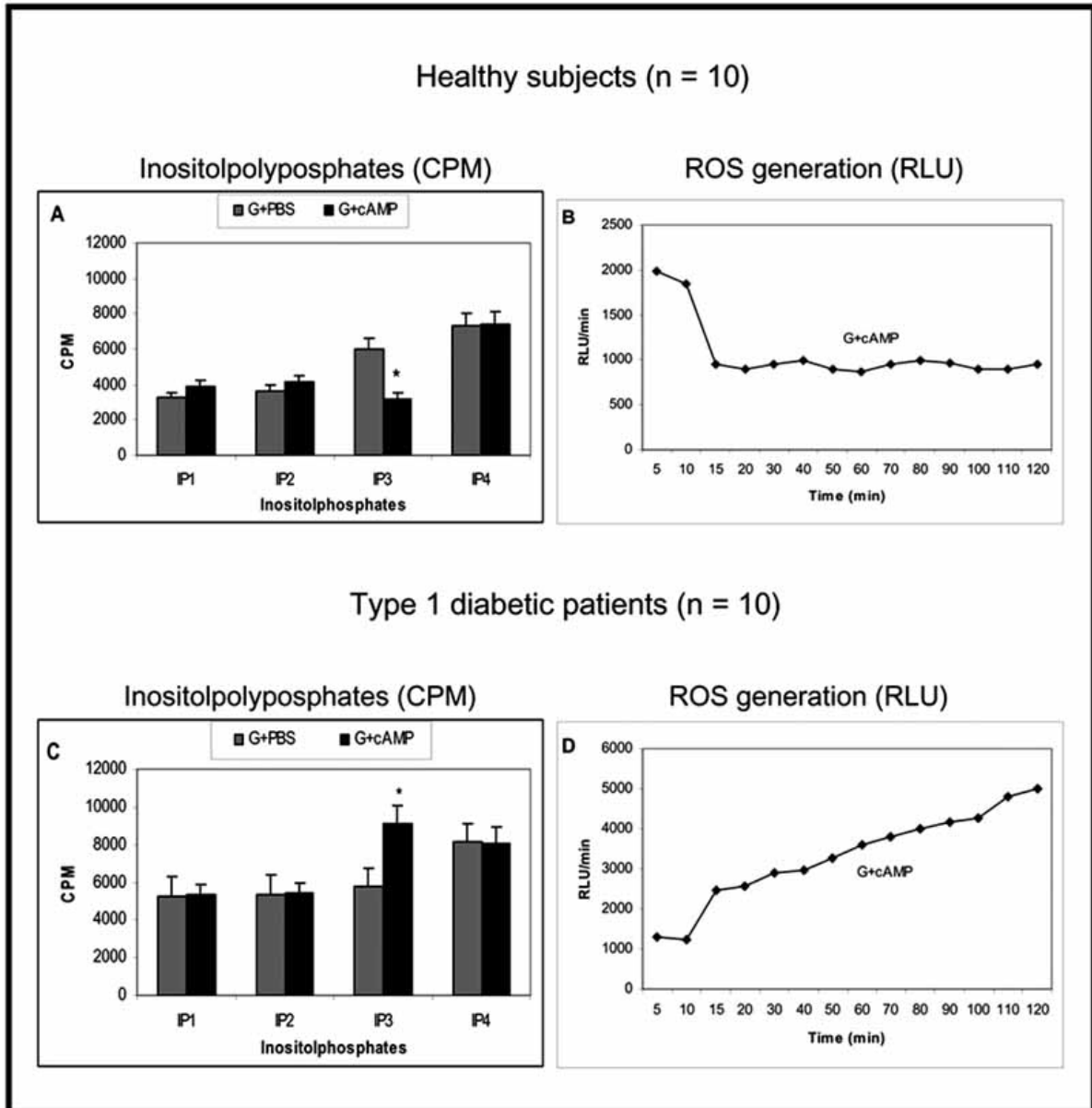
### Preparation of Granulocytes

Granulocytes were purified from 10.0 ml of heparins venous blood using a Ficoll-Hypaque gradient as previously described [13] but with minor modifications. Cellular viabil-

ities of all samples were greater than 95% as determined by the Tryphan blue exclusion test.

### Determination of ROS Production

ROS generation was measured quantitatively by a chemiluminescence assay using a luminometer (Lumat – LB 9501, EG & Berthold, Germany). An aliquot (100  $\mu$ l) of PBS containing granulocytes ( $1 \times 10^6$  cells), previously washed in PBS, was transferred to an unsealed luminescence tube together with 500  $\mu$ l of luminol (dissolved in 0.4 M dimethyl sulphoxide). The final volume was adjusted to 700  $\mu$ l with HBSS (pH 7.3). In some assays, a volume (100  $\mu$ l) of the



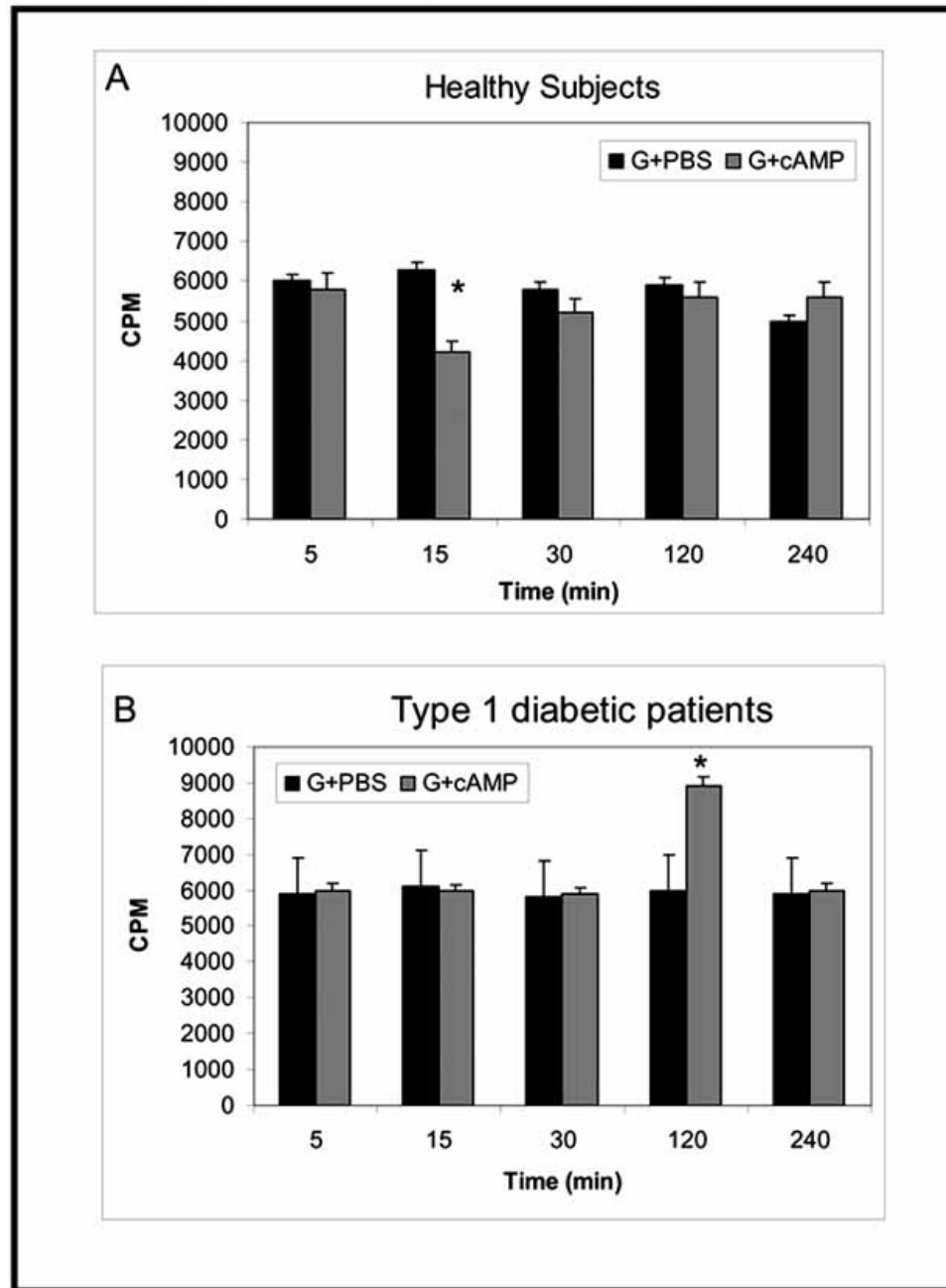
**Fig. (1).** Inverse response of  $InsP_3$  production and ROS generation by granulocytes from Type 1 diabetic patients in comparison with healthy subjects.

N = number of experiments. Reactive Oxygen Species (ROS) generation was expressed as Relative Light Units (RLU/min) during 120 minutes reaction.  $InsP_1$ ,  $InsP_2$ ,  $InsP_3$  and  $InsP_4$  were quantified by inositol- $H^3$  in a Beta Counter, and the results were expressed as count per minute (CPM). G = granulocytes; cAMP = Dibutyryl cyclic AMP, PBS = Phosphate Buffered Saline. The average  $\pm$  S.D. was compared by unpaired test Mann Whitney and \* $p < 0.05$  was considered as significant.

cAMP-elevating agent dibutyryl cAMP (dbcAMP;  $10^{-5}$  M; Sigma, St. Louis, MO, USA) was added to the incubation mixture for 120 min, before the adjustment of the final volume. The assays were carried out in duplicate and control incubations were performed simultaneously. The concentration of dbcAMP used in the assays was determined from dose-response experiments with  $10^{-2}$ ,  $10^{-5}$ ,  $10^{-6}$  and  $10^{-8}$  M of the additive: the selected concentration ( $10^{-5}$  M) produced the highest activation or inhibition of ROS production concomitant with the lowest percentage (<5%) of cell death.

### Determination of Inositolpolyphosphates Contents

Aliquots (100 $\mu$ l) containing  $1 \times 10^6$  neutrophil cells were also incubated in tubes with 1 $\mu$ Ci (1 $\mu$ l) myo-[ $^3$ H]-inositol (Amersham) and (100  $\mu$ l/ $10^{-5}$ M) dbcAMP. Final volumes were again adjusted to 250 $\mu$ l with RPMI and the tubes incubated at 37°C for 5, 15, 30, 120 and 240 min. The reaction was then stopped by placing the tubes on ice and adding 120 $\mu$ l 0.22 M HCl. Lipids were extracted by the addition of 2.7ml chloroform:methanol (1:2) and aqueous and lipid phases were separated by adding 1.8ml chloroform:water



**Fig. (2).** Kinetics studies of InsP3 release by granulocytes from healthy subjects and from Type 1 diabetic patients in the presence or in the absence of cyclic AMP-elevating agents.

Number of experiments = 10. InsP3 was quantified by inositol- $H^3$  in a Beta Counter, and the results were expressed as count per minute (CPM). G = granulocytes; cAMP = Dibutyryl cyclic AMP, PBS = Phosphate Buffered Saline. The average  $\pm$  S.D. was compared by unpaired test Mann Whitney and \* $p < 0.05$  was considered as significant.

(1:1). The water-soluble fraction (2.5ml) was diluted eight-fold with water and separated by filtration through 1.0ml DOWEX-1 columns (X8, 100-200 mesh, formate form) in Pasteur pipettes, using 25.0ml of inositol-free water, 21ml of 5.0 mM sodium tetraborate in 60mM sodium formate (10 $\mu$ g/10 $\mu$ l) (glycerophosphoinositol), 11.0 ml 0.2 M ammonium formate in 0.1 M formic acid (inositolmonophosphate), 11.0 ml 0.4 M ammonium formate in 0.1 M formic acid (inositoldiphosphate), 11.0 ml 1.0 M ammonium formate in 0.1 M formic acid (inositoltriphosphate), 11.0 ml 2.0 M ammonium formate in 0.1 M formic acid (inositoltetraphosphate), as eluants, using the method of Berridge [14]. The InsP<sub>1</sub>, InsP<sub>2</sub>, InsP<sub>3</sub> and InsP<sub>4</sub> fractions were then counted in a liquid scintillation spectrophotometer (LKB Wallac 1209 RACKBETA).

### Statistical Analysis

The statistical analysis was performed using the unpaired Mann Whitney test and the Pearson correlation test.  $p < 0.05$  was taken as the threshold of significance.

## RESULTS AND DISCUSSION

The intracellular elevation of cAMP inhibited InsP<sub>3</sub> release and ROS production in granulocytes from healthy subjects and activated in Type 1 diabetic patients (DM1). The levels of InsP<sub>1</sub>, InsP<sub>2</sub> and InsP<sub>4</sub> were not affected (Fig. 1 – panels A-D). cAMP is known to be the inhibitor of several cellular functions [15]. Our results with activation of InsP<sub>3</sub> and ROS in granulocytes from DM1 patients after cAMP elevation may suggest an adaptive metabolic response induced by chronic hyperglycemia (Fig. 1 – panels C and D). This inverse metabolic ROS-response in granulocyte appears to involve the cAMP/Epac/PKB for Type 2 diabetic patients and cAMP/PKA for healthy subjects [11,12].

The kinetics studies of InsP<sub>3</sub> release by granulocyte from healthy subjects and DM1 are shown in Fig. (2). The results showed that cells from DM1 and healthy subjects may be discriminated by the time reaction. For the diabetic cells the best time reaction was 120 minutes while for healthy subjects, 15 minutes was sufficient. The inhibitory or activatory

affects mediated by the elevation of cAMP appear to depend on intracellular calcium due to direct association observed between ROS production and InsP<sub>3</sub> release.

It is suggested that chronic hyperglycemia activates the metabolic route of DAG/PKC inducing formation of InsP<sub>3</sub>, prostanoids (PGE<sub>2</sub>) and ROS [16]. In this context, PGE<sub>2</sub>, a beta receptor activator, promotes the elevation of cAMP. Thus, hyperglycemia may induce elevation of cAMP by the action of PGE<sub>2</sub> and to induce an increase of ROS formation in granulocytes from DM1. The reaction between ROS with sugar, protein or lipids may form toxic products termed advanced glycation end products (AGEs) being associated with a pro-inflammatory response [17]. In this context, cAMP elevating agents could induce either anti-inflammatory or pro-inflammatory profiles in cells from either healthy subjects or from DM1, respectively (Table 1). InsP<sub>3</sub> release and ROS production under elevated contents of cyclic AMP exhibited different profiles for diabetic patients and healthy subjects. Our results clearly demonstrated that InsP<sub>3</sub> and ROS production in the dependence of intracellular level of cAMP are directly correlated (Table 1). The increase or decrease of both InsP<sub>3</sub> content and ROS generation by granulocytes were statically correlated only in the presence of cyclic AMP. In granulocytes from healthy subjects, InsP<sub>3</sub> and ROS decreased at 15 minutes reaction ( $p < 0.05$ ;  $r = 0.9661$ ) while for granulocytes DM1 both InsP<sub>3</sub> and ROS generation increased at 120 minutes reaction ( $p < 0.05$ ;  $r = 0.9884$ ) (Table 1). In contrast, in the presence of cAMP, at 120 minutes reaction for healthy subjects and 15 minutes for DM1, no correlation was observed ( $r = 0.0478$ ;  $r = 0.0012$ ). Time reaction was important and appears to be dependent on the metabolic status of the cells. There was no correlation however in the absence of cyclic AMP between InsP<sub>3</sub> and ROS production. The observed delay and activation inversion of metabolic response in diabetic cells in the dependence of cAMP demonstrated to be a possible effect of hyperglycemia on cellular response. Thus, the activation of InsP<sub>3</sub> and ROS due to the elevation of cAMP in conjunction with similar results with NO [5, 18] suggested a pro-inflammatory profile for granulocytes from DM1 patients. It may have consequences on vascular damage and innate immunity in diabetes. Further studies are needed to identify other signaling pathways with

**Table 1. Correlation between InsP<sub>3</sub> Release and ROS Generation by Granulocytes from Healthy Subjects and Type 1 Diabetic Patients in the Presence or in the Absence of Cyclic AMP-Elevating Agents**

Time Reaction	Studied Parameters	Healthy subjects			Type 1 diabetic patients		
		G+PBS	p	G+cAMP	G+PBS	p	G+cAMP
15	IP3	5600 $\pm$ 523	<b>p&lt;0.05</b>	3709 $\pm$ 214	2933 $\pm$ 125	<b>N.S</b>	3005 $\pm$ 369
		<b>r = 0.2195</b>		<b>r = 0.9661*</b>	<b>r = 0.0062</b>		<b>r = 0.0012</b>
	ROS	4269 $\pm$ 325	<b>p&lt;0.05</b>	2534 $\pm$ 198	3504 $\pm$ 239	<b>N.S</b>	3740 $\pm$ 398
120	IP3	4872 $\pm$ 425	<b>N.S</b>	4836 $\pm$ 369	5685 $\pm$ 539	<b>p&lt;0.05</b>	9193 $\pm$ 854
		<b>r = 0.1700</b>		<b>r = 0.0478</b>	<b>r = 0.0020</b>		<b>r = 0.9884*</b>
	ROS	1128 $\pm$ 185	<b>N.S</b>	938 $\pm$ 98	3848 $\pm$ 356	<b>p&lt;0.05</b>	5118 $\pm$ 458

Number of experiment = 10. The average  $\pm$  S.D. was compared by unpaired test Mann Whitney and  $p < 0.05$  was considered as significant. The Person correlation was used for comparing evolution of InsP<sub>3</sub> release and ROS production. \* = significant correlation between InsP<sub>3</sub> and ROS generation. InsP<sub>3</sub> and ROS were expressed as CPM  $\pm$  SD and RLU/min  $\pm$  SD, respectively.

altered metabolic response in leukocytes from diabetic patients.

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