Modulation of the Reactive Oxygen Species (ROS) generation mediated by cyclic AMP-elevating agents or Interleukin 10 in granulocytes from type 2 diabetic patients (NIDDM): a PKA-independent phenomenon

Summary-Background: The present study investigates the hypothesis that cells from ill patients and from healthy subjects may have different reactivity under metabolic stimulation as a consequence of an disease-induced metabolic adaptation.

Methods: Granulocytes either from healthy subjects or from type II — Non Insulin Dependent Diabetes Mellitus (NIDDM) patients were compared in their capacities to generate Reactive Oxygen Species (ROS). The ROS generation was comparatively determined in a chemiluminescence assay, luminol-dependent, after cell incubation in the presence of either cyclic AMP — elevating agents or Interleukin 10. In some experiments the cells were pretreated with H89 compound (a PKA inhibitor) or with diphenylene iodonium (DPI), a NADPH-oxidase inhibitor.

Results: Our results showed an increased ROS generation in granulocytes from diabetic patients in absence of cyclic AMP-elevating agents or IL-10. In the presence of cyclic AMP-elevating agents was observed an inverse metabolic response in granulocytes from diabetic patients in comparison to cells from healthy subjects. The granulocytes were preincubated in the presence of cyclic AMP-elevating agents — amminophylline (AMF) or dibutyryl cyclic AMP (dbcAMP) — or interleukin 10 (IL-10). The AMF, dbcAMP and IL-10 inhibited ROS production by granulocytes from healthy subjects. By contrast, AMF and dbcAMP activated cells from diabetic patients while IL-10 had no effect. The inhibition of ROS induced by AMF, dbcAMP or IL-10 was promptly abolished by the pretreatment of the cells with either PKA H89 inhibitor or NADPH-oxidase inhibitor (DPI) in granulocytes from healthy subjects. In relation to the granulocytes from type 2 diabetes patients, the activation of ROS generation mediated by AMF and dbcAMP was fully abolished by NADPH-oxidase DPI-inhibitor, but not by PKA H89 inhibitor.

Conclusions: Our present results reinforce the hypothesis that cells from ill patients (type II diabetic) when compared to cells from healthy subjects have different reactivity under metabolic stimulation. ROS production by human granulocytes is modulated by cyclic AMP-elevating agents and IL-10. The inhibition of the ROS production in granulocytes from healthy subjects was PKA-dependent while the activation in granulocytes from patients is PKA-independent. Possible cAMP/Epac/PKB-dependent. The correlation between activation of ROS production in granulocytes from diabetic patients and pathogenesis of diabetes can be suggested, however, further and extensive studies are needed for demonstrating this suggestion.

Key-words: Diabetes · Cyclic AMP · Granulocytes · ROS · PKA · NADPH-oxidase.

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Modulation de la production d’espèces radicales de l’oxygène médiée par des agents élevant l’AMPc ou par l’interleukine-10 dans des granulocytes de patients diabétiques de type 2 : un phénomène indépendant de la PKA

Résumé-état de la question : Cette étude teste l’hypothèse selon laquelle des cellules de patients malades et de sujets sains ont une réactivité différente sous une stimulation métabolique, comme conséquence d’une adaptation métabolique induite par la maladie.

Méthodes : Des granulocytes de sujets sains et de diabétiques de type 2 (NIDDM) ont été comparés dans leur capacité à générer des espèces radicales de l’oxygène (ROS). La génération de ROS a été déterminée de façon comparative dans un test de chemiluminescence, luminol-dépendant, après incubation cellulaire en présence d’agents élevant l’AMPc ou d’interleukine-10. Dans certaines expériences, les cellules ont été prétraitées par le composé H89 (inhibiteur de la PKA) ou par diphenylene iodonium (DPI), inhibiteur de la NADPH-oxidase.

Résultats : Nos résultats montrent une augmentation de la production de ROS dans les granulocytes de patients diabétiques en l’absence d’agents élevant l’AMPc ou d’IL-10. En présence d’agents élevant l’AMPc, on observe une réponse métabolique inverse dans les granulocytes de patients diabétiques par rapport aux cellules de sujets sains. Les granulocytes ont été préincubés en présence d’agents élevant l’AMPc — amminophylline (AMF) ou dibutyryl cyclic AMP (dbcAMP) — ou d’interleukine 10 (IL-10). AMF, dbcAMP et IL-10 inhibent la production de ROS par les granulocytes de sujets sains. À l’inverse, AMF et dbcAMP activent les cellules de patients diabétiques, tandis qu’IL-10 n’a pas d’effet. L’inhibition de la production de ROS induite par AMF, dbcAMP ou IL-10 est rapidement abolie par le prétraitement des cellules avec soit l’inhibiteur de la PKA (H89) soit l’inhibiteur de la NADPH-oxidase (DPI) dans les granulocytes de sujets sains. Dans les granulocytes de diabétiques de type 2, l’activation de la génération de ROS médieée par AMF et dbcAMP est totalement abolie par l’inhibiteur de la NADPH-oxidase (DPI), mais par l’inhibiteur de la PKA (H89).

Conclusions : Nos résultats renforcent l’hypothèse selon laquelle les cellules de patients diabétiques de type 2, par rapport aux cellules de sujets sains, ont une réactivité différente après stimulation métabolique. La production de ROS par les granulocytes humains est modulée par les agents élevant l’AMPc et par l’IL-10. L’inhibition de la production de ROS dans les cellules de sujets sains est PKA-dépendante tandis que l’activation dans les granulocytes de patients est PKA-indépendante. La réponse métabolique inverse, dans les cellules de patients, suggère le recours à une voie métabolique alternative PKA-indépendante, possiblement AMPc/Epac/PKB-dépendante. Une correlation entre l’activation de la production de ROS dans les granulocytes de patients diabétiques et la pathogénie du diabète peut être évoquée, mais nécessite des études supplémentaires pour être démontrée.

Mots-clés : Diabète · AMP cyclique · Granulocytes · ROS · PKA · NADPH-oxidase.
Diabetes is a multifactorial disease with alteration and adaptation in the glucose metabolism. It has been suggested that hyperglycemia conditions may alter signaling pathways such as, impaired ability of insulin to activate PKB/Akt; increased diacylglycerol synthesis; activation of AMP-activated protein Kinase; mitogen-activated protein kinase (MAPKs) and JAK/STAT signaling system [1, 2]. The increased Reactive Oxygen Species (ROS) (oxidative stress) interfere with signaling pathways by activating JNK and p38 kinases, which are involved in signaling cascade of PI-3K [3]. We have demonstrated that ROS production is inhibited by cyclic AMP-elevating agents and IL-10 [4]. Nitric oxide generation in granulocytes from type II diabetic patients is activated by cAMP and inhibited by cGMP, showing an inverse metabolic response in comparison to granulocytes from healthy subjects [5]. In the present paper, we have studied, comparatively, the effect of cyclic AMP-elevating agents and IL-10 on ROS generation in granulocytes from type II diabetic patients and from healthy subjects and the possible involvement of PKA and NADPH-oxidase enzymatic system in the ROS modulation in diabetes.

Materials and methods

Reagents

Dibutyryl cyclic AMP, PKA inhibitor H89 and DPI (diphenylene iodonium) were purchased from Sigma Co., Aminophylline from Sandoz, Interlukin 10 (IL-10) (DNAX).

Granulocyte Separation

The granulocytes were purified from 10.0 ml of heparinized venous blood using the Ficoll-Hypaque gradient according to Bicalho et al., [6] with slight modifications. The cellular viability of each sample was always greater than 95% as determined by the Trypan blue exclusion test.

Diabetic Patients

The Ethical Committee of Santa Casa Hospital of Belo Horizonte — Brazil approved this study and the informed consent was obtained from all participants. All of the volunteers were submitted to detailed physical examination, evaluation of medical history and laboratory data, before entering in the study. Fifty-two non-diabetics controls and sixty-three type II diabetic patients were selected by Hemominas Foundation and by Dr. Ataualpa Pereira Reis (CLIMED). The type II diabetic patients 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), respectively. The inclusion criteria were: I) age ranging from 45 to 70 years old; II) glucose level equal or exceeds 140 mg/dl on at least two separated occasions and; III) glucose level greater than 200mg/dL in a 2-hours oral glucose tolerance test (OGTT); IV) non-insulin requiring and V) taking pills of chlorpropamide. The population of patients was from type II NIDDM classified as A and B, based on BMI which was 28.0 ± 0.7 (ranging from 25 to 32). The laboratories and clinical tests showed: blood pressure = 132/85 (from 120/80 to 144/91); glycemic control (gHb) = 8.2 ± 0.7 (7 to 9%); total cholesterol = 248 ± 2.7 (230 to 262 mg/dL); triglicerides = 169.5 ± 6.4 [150 to 200 mg/dL]; serum creatinin=1.35 ± 0.09 [0.9 to1.9 mg/dL]. Retinopathy, neuropathy and nephropathy, in several grades of severity were diagnosticated in 72, 55 and 80% of the patients. The time of duration of diabetes was 4.7 ± 0.7 (1 to 9 years) (Tab I).

Determination of ROS production

The quantitative ROS determination was performed in a chemiluminescence assay, by the incubation of granulocytes

Table I

<table>
<thead>
<tr>
<th>Age group</th>
<th>Males</th>
<th>Females</th>
<th>Total</th>
<th>Means age years old ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Non-diabetic controls</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>45-70 years old</td>
<td>13</td>
<td>17</td>
<td>30</td>
<td>58.5 ± 13.0</td>
</tr>
<tr>
<td><strong>Type 2 diabetic patients</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>45-70 years old</td>
<td>14</td>
<td>16</td>
<td>30</td>
<td>58.8 ± 15.0</td>
</tr>
</tbody>
</table>

The age groups were comparable in size and sex distribution.

three type II diabetic patients were excluded based on the exclusion criteria by presenting one or more of these conditions and/or pathologies: smokers, hypertension treatment, cardiac insufficiency, pregnancy, alcoholism, dementia, inflammation, malignance disease, infection or fasting plasma glucose > 110 < 140 mg/dl. The healthy non-diabetic controls were formed by 30 people (17 women and 13 men) and they were selected by Hemominas Foundation and by Dr. Ataualpa Pereira Reis (CLIMED). The type II diabetic patients 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), respectively. The inclusion criteria were: I) age ranging from 45 to 70 years old; II) glucose level equal or exceeds 140 mg/dl on at least two separated occasions and; III) glucose level greater than 200mg/dL in a 2-hours oral glucose tolerance test (OGTT); IV) non-insulin requiring and V) taking pills of chlorpropamide. The population of patients was from type II NIDDM classified as A and B, based on BMI which was 28.0 ± 0.7 (ranging from 25 to 32). The laboratories and clinical tests showed: blood pressure = 132/85 (from 120/80 to 144/91); glycemic control (gHb) = 8.2 ± 0.7 (7 to 9%); total cholesterol = 248 ± 2.7 (230 to 262 mg/dL); triglicerides = 169.5 ± 6.4 [150 to 200 mg/dL]; serum creatinin=1.35 ± 0.09 [0.9 to1.9 mg/dL]. Retinopathy, neuropathy and nephropathy, in several grades of severity were diagnosticated in 72, 55 and 80% of the patients. The time of duration of diabetes was 4.7 ± 0.7 (1 to 9 years) (Tab I).

Determination of ROS production

The quantitative ROS determination was performed in a chemiluminescence assay, by the incubation of granulocytes
(1 × 10⁶ cells/100 µl Hank’s balanced salt solution, HBSS) either with 100 µl of cyclic AMP (dibutyryl cyclic AMP), of amminophylline (AMF), or with 10 µg/µl of interleukin 10 (IL-10). All reagents were used at 10⁻⁴ M except IL-10 which were used at 10 µg/5 µl. Each unscaled luminescence tube received an additional 500 µl of luminol (dissolved in dimethyl sulfoxide 0.4 M) and the final volume was adjusted to 700 µl with HBSS (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 45 min, time enough for observation of the peak. The results were expressed in RLU/min (Relative Light Unit per minutes). The control experiments were done simultaneously. The concentration of 10⁻⁴ M used for experiments with cyclic AMP elevating-agents was based on 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 lower percentage of dead cells (<5%). In some experiments, granulocytes were pre-incubated in the presence of either PKA (H89) (1 µM) or NADPH-oxidase inhibitors [DPI — (diphenylene iodonium)] (10 µM) for 30 minutes. The granulocytes were washed and cyclic AMP-elevating agents or IL-10 were added.

**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.

**Results**

We have compared the capacity of granulocytes from non-diabetic subjects and from type II diabetic patients (NIDDM) in producing ROS in the presence or in the absence of dibutylryl cyclic AMP (dbcAMP); amminophylline (AMF) (a cyclic AMP phosphodiesterase blocker) or interleukin 10 (IL-10). The results are shown in the Figure 1 and Table II. The ROS production by granulocytes in the absence of cAMP-elevating agents or IL-10, expressed as Relative Light Units (RLU) during 45 min. reaction was 2034 ± 195 for non-diabetes subjects and 3732 ± 321 for type II diabetes patients (NIDDM) (p < 0.05) (Tab II). Granulocytes from type II diabetic patients (NIDDM) produced ROS 1.84 times than that observed with non-diabetic subjects, suggesting an activated ROS producing metabolism possibly disease-induced. This spontaneously ROS production was promptly blocked by DPI (NADPH-oxidase inhibitor) in both granulocytes from healthy subjects and from patients (Tab II). The percentage of inhibition (Tab II) was 94.0 and 93.2 for granulocytes from healthy subjects or diabetic patients, respectively. The H89 compound (PKA inhibitor) inhibited ROS generation in cells from healthy subjects (percentage of inhibition = 39.5), but had no significant effect on granulocytes from diabetic patients (Tab II).

ROS production by granulocytes from non-diabetic subjects was inhibited by dbcAMP, AMF and IL-10 [G + dbcAMP =784 ± 69; G + AMF = 832 ± 82; G + IL-10 = 632 ± 59], in comparison to the control G+ HBSS = 2034 ± 195 [line 1]. An inverse metabolic ROS response was observed with granulocytes from type II diabetic patients [G + dbcAMP = 15,983 ± 1621; G + AMF = 12,843 ± 1302] (Tab II and Fig 1). Interleukin 10 did not inhibit ROS production in cells from diabetic patients (NIDDM) [G + IL-10 = 3859 ± 365] in comparison to the diabetic control in the absence of IL-10 [G + HBSS = 3732 ± 321] (p > 0.05) (Tab II and Fig 1).

In order to investigate the biochemical events involved in this altered granulocyte reactivity, we tested the effect of PKA and NADPH-oxidase inhibitors. The H89 compound (PKA inhibitor) and diphenylene iodonium (DPI), a NADPH-oxidase inhibitor were used. The results are shown in the (Tab II). The ROS production inhibition in-

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**Figure 1**

Comparative effect of cyclic AMP-elevating agents and Interleukin 10 on Reactive Oxygen Species (ROS) generation by granulocytes from healthy subjects and from type II diabetic patients (NIDDM). HBSS: Hank’s balanced Salt Solution; dbcAMP: dibutyryl cyclic AMP; AMF: amminophylline; IL-10: Interleukin 10; RLU/45min: Relative Light Units during 45 minutes reaction; *: significant at p < 0.05 by Student “t” test in relation to the respective control.
Table II
Effect of the inhibition of PKA and NADPH-oxidase on the ROS production by granulocytes from diabetic patients.

Total ROS generation-RLU/45min

<table>
<thead>
<tr>
<th>Experiments</th>
<th>Non-Diabetic Subjects</th>
<th>Type II diabetic patients</th>
</tr>
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<tbody>
<tr>
<td>1-G + HBSS</td>
<td>2034 ± 195</td>
<td>3732 ± 321</td>
</tr>
<tr>
<td>2-G + HBSS + DPI</td>
<td>523 ± 65</td>
<td>724 ± 52</td>
</tr>
<tr>
<td>3-G + HBSS + H89</td>
<td>1232 ± 104</td>
<td>3569 ± 296</td>
</tr>
<tr>
<td>4-G + IL-10</td>
<td>632 ± 59*(N.S)</td>
<td>3859 ± 365(N.S)</td>
</tr>
<tr>
<td>5-G + H89 + IL-10</td>
<td>2624 ± 30(N.S)</td>
<td>3692 ± 142(N.S)</td>
</tr>
<tr>
<td>6-G + DPI + IL-10</td>
<td>89 ± 7*(N.S)</td>
<td>292 ± 25*(N.S)</td>
</tr>
<tr>
<td>7-G + dbcAMP</td>
<td>784 ± 69*</td>
<td>15983 ± 1621*</td>
</tr>
<tr>
<td>8-G + H89 + dbcAMP</td>
<td>2324 ± 142(N.S)</td>
<td>16082 ± 1162*</td>
</tr>
<tr>
<td>9-G + DPI + dbcAMP</td>
<td>72 ± 6*(N.S)</td>
<td>1492 ± 102(N.S)</td>
</tr>
<tr>
<td>10-G + AMF</td>
<td>832 ± 82*</td>
<td>12843 ± 1302*</td>
</tr>
<tr>
<td>11-G + H89 + AMF</td>
<td>2231 ± 163(N.S)</td>
<td>15242 ± 1232(N.S)</td>
</tr>
<tr>
<td>12-G + DPI + AMF</td>
<td>69 ± 5*(N.S)</td>
<td>1329 ± 111*(N.S)</td>
</tr>
</tbody>
</table>

RLU/45min: Relative Light Units during 45 minutes reaction; *values significant at p < 0.05 by Student “t” test when compared to control in the line 1; NS: not significant; cAMP: dibutyryl cyclic AMP; AMF: Amminophylline; IL-10: Interleukin 10; H89: PKA inhibitor; DPI (DPI — (diphenylene iodonium)): NADPH-oxidase inhibitor.

The oxidative burst ROS producing is enhanced by TNF-α, GM-CSF, IL-8, IL-1, IL-6 and inhibited by cyclic AMP-elevating agents and IL-10 [4, 9-11].

Our present results clearly demonstrate that cyclic AMP-elevating agents may modulate ROS generation in granulocytes from non-diabetic subjects and from type II diabetic patients (NIDDM). Interleukin 10 was able to modulate ROS production in cells from non-ill subjects but had no effect on granulocytes from diabetic patients. The ROS generation by granulocytes from non-diabetic subjects was inhibited by dibutyryl cyclic AMP, amminophylline and IL-10. However, with cells from diabetic patients, was observed an altered metabolic ROS response (Fig 1 and Tab II). In this case, cyclic AMP-elevating agents (dibutyryl cAMP and Amminophylline) strongly activate the ROS production, but IL-10 has no effect (Tab II).

For connecting the altered metabolic ROS response detected in granulocytes from type II diabetic patients to ROS generation, specific inhibitions of Protein Kinase A (PKA) and NADPH-oxidase were tested. For inhibition assay were used, respectively, the H89 compound, a PKA inhibitor and diphenylene iodonium (DPI), a NADPH-oxidase inhibitor. The pretreatment of granulocytes with DPI abolished the spontaneous ROS production in both granulocytes from healthy and from ill patients (Tab II). However, H89 compound was inhibitory for granulocytes from healthy subjects but not for cells from diabetic patients (Tab II). These data suggest a dependence of PKA for ROS producing in cells from healthy subjects but not in cells from diabetic patients. The ROS production in cells from diabetic patients was significantly greater than that with cells from healthy subjects, suggesting an exacerbation in the NADPH-oxidase activity. The effective action of NADPH-oxidase in ROS producing was PKA-dependent in granulocytes from healthy subjects but not in cells from type II diabetic patients (Tab II). The spontaneous increased ROS production by granulocytes from diabetic patients (Tab II) may suggest an oxidative stress disease-induced. It is accepted that oxidative stress is important for induction of premature atherosclerose and it is also known that diabetes is a major risk factor for vascular disease.

Cyclic AMP-elevating agents inhibited or activated ROS production in granulocytes either from healthy subjects or from type II diabetic patients, respectively Figure 1 and Table II. The pretreatment of granulocytes from healthy subjects with 1µM of H89 abolished the inhibitory effect mediated by the elevation of cAMP (Tab II), suggesting that ROS production in the presence of increased level of cAMP depends on PKA pathway for inhibiting NADPH-oxidase system in the ROS generation. By contrast, the activation of ROS production in granulocytes from type II diabetic patients was not altered by H89 compound, suggesting a PKA-independent pathway in cells from diabetic patients. The effect induced by IL-10 on cells from diabetic patients was not affected by the H89 compound. These results (Tab II) suggest that the intracellular elevation of cyclic AMP may induce strong alteration on oxidative metabolism of the granulocytes from type II diabetic patients (NIDDM).

Thus, our results suggest that granulocytes from patients react differently under stimulation with cyclic AMP-
elevating agents in a cAMP/PKA-independent pathway. In this context, it has been suggested the multiple cAMP mediated pathways exist and only some are PKA-dependent [12-14]. Recently was discovered a new cAMP receptor — Epac — and it represents an important piece to our understanding of cAMP signaling. Both cAMP receptors — PKA and Epac — mediate opposing effect on PKB. Mei et al. [12] have proposed a model for cAMP-mediated PKB. On the one hand, cAMP acting through PKA can inhibit PKB activity. On the other hand, cAMP can also activate PKB through an Epac-mediate signaling pathway, PI-3K-dependent manner. Since cAMP can either inhibit or stimulate PKB activity, it is conceivable that Epac and PKA acting as downstream receptors, may differentially mediate these opposing effect on ROS generation observed in granulocytes from healthy subjects and from ill patients. It is also accepted that PKB is involved in insulin signaling and diabetes [15].

Thus, our results demonstrated that spontaneous ROS generation in granulocytes from patients is PKA-independent and NADPH-oxidase dependent (Tab II). These present results in conjunction with other previously reported in relation to NO in diabetes [5] reinforce our suggestion that granulocytes from type II (NIDDM) diabetic patients, in the presence of increased level of intracellular cAMP, have an altered and inverse metabolic response for producing oxidizing species from oxygen (ROS) and from nitrogen (NO).

Our results clearly demonstrate that granulocytes from type II patients are spontaneously up-regulated for ROS producing in a PKA-independent pathway and suggest that under increased cAMP, granulocytes from healthy subjects use the cAMP/PKA dependent pathway while cells from patients is PKA-independent, possibly using the cAMP/Epac/PKB-dependent pathway. The differential and inverse granulocyte reactivity may be a reflex of an altered signaling pathway induced by the disease. This intricate modulation phenomenon needs further and extensive investigations to understand the modulation of ROS production by cyclic nucleotides and its role in the pathological complication induced by diabetes.

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References