Modulation of the production of Reactive Oxygen Species (ROS) by cAMP-elevating agents in granulocytes from diabetic patients: an Akt/PKB-dependent phenomenon

JA Nogueira-Machado¹, FC Lima e Silva¹, EP Cunha¹, MR Calsolari¹, DC Costa², CS Perilo², BC Horta², IC Ferreira², MM Chaves²

SUMMARY

Background: Granulocytes from healthy subjects and from patients suffering from diabetes mellitus present differences in reactivity to stimulation with cyclic nucleotide-elevating agents. The production of reactive oxygen species (ROS) is inhibited in cells from non-diabetic subjects following such stimulation, but activated through a PKA-independent signaling pathway in granulocytes from type 1 and type 2 diabetic patients. The aim of the present study was to understand better the changes in signaling mechanisms induced by the disease.

Methods: ROS production in granulocytes from healthy subjects and from type 1 and type 2 diabetic patients was measured using a luminol-dependent chemiluminescence assay. Granulocytes were stimulated by the addition of the cAMP-elevating agent dibutyryl cAMP. In some experiments, granulocytes were pre-treated with an inhibitor of PKA or Akt/PKB prior to cAMP stimulation.

Results: Intracellular elevation of cAMP induced a PKA-dependent and Akt/PKB-independent inhibition of ROS production in granulocytes from healthy subjects, but a significant activation in cells from both type 1 and type 2 diabetic patients. Most significantly, activation of ROS generation in cells from diabetic patients was shown to be Akt/PKB-dependent and PKA-independent.

Conclusions: These results suggest that chronic hyperglycaemia could induce metabolic adaptation in cAMP-related signaling mechanisms. Epac (exchange protein directly activated by cAMP) is a novel cAMP receptor besides PKA involved in different signaling pathways. The cAMP-stimulated inverse ROS response in granulocytes from type 1 and type 2 diabetic patients may be due to a change in signaling pathways from cAMP/PKA to cAMP/Epac/Akt/PKB. These preliminary results require further studies in order to evaluate their consequences on innate immunity and pathogenesis of diabetes mellitus.

Key-words: Diabetes mellitus · cAMP · Oxidative stress · Reactive oxygen species · Granulocytes · Akt/PKB · PKA.

RÉSUMÉ

Modulation de la production de radicaux libres par les granulocytes de patients diabétiques, médidée par des agents élevant l’AMPc, phénomène dépendant de la Akt/PKB.

Objectif : Les granulocytes de patients diabétiques ont une réactivité différente de celle des non diabétiques lors de la stimulation par des substances qui élevent les concentrations intracellulaire d’AMP cyclique (AMPc). La production d’espèces réactives de l’oxygène (ROS) est inhibée par l’AMPc dans les cellules issues de non-diabétiques, mais est activée par une voie de signalisation indépendante de la PKA dans les cellules de diabétiques de type 1 et 2. Le but de cette étude était de préciser les anomalies de signalisation liées au diabète.

Méthodes : La production de ROS par les leucocytes de témoins non-diabétiques et de diabétiques de type 1 et 2 a été mesurée par une méthode de chemiluminescence luminol-dépendante, après incubation en présence d’agents élevant l’AMPc. Dans certains expériences, les cellules ont été prétraitées par un inhibiteur de la PKA (H89) ou par inhibiteur de la Akt/PKB avant stimulation de l’AMPc.

Résultats : L’augmentation intracellulaire d’AMPc entraîne une inhibition de la production de ROS dépendant de la PKA et indépendante de l’Akt/PKB dans les cellules provenant des non-diabétiques. L’inhibition induite par l’AMPc est abolie par le prétraitement des cellules par l’H89. En revanche, une activation de la production de ROS est observée dans les granulocytes de patients diabétiques, indépendante de la PKA et dépendant de l’Akt/PKB. La production de ROS par les cellules de patients diabétiques est abolie par un prétraitement par inhibiteur de l’Akt/PKB.

Conclusions : Ces résultats suggèrent que l’hyperglycémie chronique pourrait induire une adaptation métabolique des voies de signalisation liées à l’AMP cyclique. L’Epac (protéine échangeur directement activée par l’AMPc) est un nouveau récepteur de l’AMPc à côté de la PKA, impliqué dans différentes voies de signalisation. L’inversion de la réponse des ROS stimulée par l’AMPc dans les cellules de patients diabétiques de type 1 et 2 pourrait être liée à un changement de voie de signalisation AMPc/Epac/Akt/PKB au lieu de AMPc/PKA. Ces résultats préliminaires nécessitent des travaux ultérieurs pour en évaluer les conséquences vis à vis de l’immunité et de la physiopathologie du diabète.

Mots-clés : Diabète sucré · Stress oxydatif · Granulocytes · Espèces réactives de l’oxygène · PKA · Akt/PKB · AMP cyclique.

Address correspondence and reprint requests to:
JA Nogueira-Machado. Núcleo de Pesquisa e Pós-Graduação (NPPG), 90 andar ala D, Hospital Santa Casa de Belo Horizonte, Av. Francisco Sales 1111, Santa Efigênia, 30150-221, Belo Horizonte, Minas Gerais, Brasil. aunog.bh@terra.com.br or nogueira.machado@pesquisador.cnpq.br

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Diabetes mellitus, a chronic disease characterised by high blood glucose levels, is classified into two major forms, namely, type 1 and type 2. It is accepted that diabetic hyperglycaemia leads to alterations and adaptations in several of the biochemical signaling pathways [1-4] and causes a variety of pathological complications [5].

We have previously suggested [6] that the chronic hyperglycaemic conditions associated with type 2 diabetes induce alterations in the reactivity of the granulocytes. Both cAMP and cGMP have been shown to elicit inverse metabolic responses in the generation of nitric oxide in granulocytes from such patients compared with those observed in healthy subjects [6]. Similar effects have recently been demonstrated with respect to the production of reactive oxygen species (ROS) following stimulation of granulocytes with cAMP-elevating agents [7]; this inverse ROS metabolic response was postulated to be PKA-independent. The aim of the present study was to evaluate the roles of the cAMP/PKA and Akt/PKB signaling pathways in cAMP-induced ROS production in granulocytes from type 1 and type 2 diabetic patients in comparison with those from non-diabetic subjects.

Subjects and methods

Subjects

Details of the study were presented to and approved by the Ethics Committee of the Hospital Santa Casa de Belo Horizonte (Belo Horizonte – MG, Brazil) and appropriate informed consent was obtained from all participants. Each volunteer was submitted to a detailed physical examination, as well as to an evaluation of medical history and laboratory data, before being considered for the study. Thirty healthy subjects, who were using no medications were selected by two of us. It is accepted that diabetic hyperglycaemia leads to alterations and adaptations in several of the biochemical signaling pathways [1-4] and causes a variety of pathological complications [5].

Preparation of granulocytes

Granulocytes were purified from 10.0 ml of haphazardly selected venous blood using a Ficoll-Hypaque gradient as previously described [8]. Briefly, we have used three different densities of Ficoll-Hypaque gradient and three interfaces were formed after centrifugation. The first interface (from top to the bottom) was mononuclear cells-rich and depleted of granulocytes; the second was neutrophil-rich (100%) and the third interface was composed by neutrophils (±95%) and eosinophils (±5%). In our experiments we have used a mixture of second and third interfaces rich in granulocytes (100%) and depleted of mononuclear cells. Cellular viabilities of all samples were greater than 95% as determined by the Trypan blue exclusion test.

Determination of the production of ROS

ROS generation was measured quantitatively by chemiluminescence assay using a luminometer (Lumat – LB 9501, EG & Berthold, Germany). An aliquot (100 ml) of Hank’s balanced salt solution (HBSS) containing granulocytes (1 x 10^6 cells), previously washed in PBS, was transferred to an unaltered luminescence tube together with 500 ml of luminol (dissolved in 0.4 M dimethyl sulphoxide). The final volume was adjusted to 700 ml with HBSS (pH 7.3). In some assays, a volume (100 ml) of the cAMP-elevating agent dibutyryl cAMP (dbcAMP; 10^-3 M; Sigma, St. Louis, MO, USA) was added to the incubation mixture before adjustment of the final volume. Incubations were performed at 37°C and chemiluminescence [expressed in relative light units (RLU)/min] was recorded over a 60 min period to ensure complete determination of the peak. All assays were carried out in duplicate and control incubations were performed simultaneously. In some experiments, granulocytes were pre-incubated for 30 min with either 1 µM H89 (a PKA inhibitor; Sigma) or 1 µM Akt/PKB inhibitor (Calbiochem, San Diego, CA, USA) after which the granulocytes were washed in PBS and dbcAMP added.

Table I

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Non-diabetic</th>
<th>Type 1</th>
<th>Type 2*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>36±12</td>
<td>34±15</td>
<td>60±13</td>
</tr>
<tr>
<td>N (M/F)</td>
<td>30 (17/13)</td>
<td>7 (4/3)</td>
<td>22 (12/10)</td>
</tr>
<tr>
<td>Retinopathy</td>
<td>–</td>
<td>2/7</td>
<td>5/22</td>
</tr>
<tr>
<td>Neurupathy</td>
<td>–</td>
<td>1/7</td>
<td>1/22</td>
</tr>
<tr>
<td>Nephropathy</td>
<td>–</td>
<td>2/7</td>
<td>3/22</td>
</tr>
<tr>
<td>Diabetes duration</td>
<td>–</td>
<td>8.0±1.2</td>
<td>5.4±0.6</td>
</tr>
<tr>
<td>Insulin requirement</td>
<td>–</td>
<td>Yes</td>
<td>–</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.8±2.3</td>
<td>24.7±1.5</td>
<td>29.4±1.7</td>
</tr>
<tr>
<td>Fasting plasma glucose (mmol/l)</td>
<td>4.5±0.48</td>
<td>10.17±1.04</td>
<td>9.90±0.99</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.3±0.7</td>
<td>8.4±0.8</td>
<td>7.9±0.5</td>
</tr>
<tr>
<td>Anti-GAD Antibodies</td>
<td>–</td>
<td>Yes</td>
<td>–</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>4.79±0.03</td>
<td>6.09±0.06</td>
<td>6.48±0.06</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.69±0.01</td>
<td>2.15±0.02</td>
<td>1.86±0.02</td>
</tr>
<tr>
<td>Serum Creatinine</td>
<td>57.2±0.9</td>
<td>79.2±1.8</td>
<td>1.2±0.15</td>
</tr>
</tbody>
</table>

* Type 2 diabetic patients were taking metformin.
as appropriate. The concentration of dbcAMP employed in the assays was determined from dose–response experiments with 10⁻², 10⁻⁴, 10⁻⁶ and 10⁻⁸ M of the additive: the selected concentration (10⁻⁴ M) produced the highest activation or inhibition of ROS production concomitant with the lowest percentage (<5%) of cell death.

Statistical analysis

Data are expressed as mean ±SD values. The statistical analysis was performed using paired and unpaired Student “t” test. A P<0.05 value was considered as significant.

Results

Modulation of the production of ROS by inhibitors of PKA or Akt/PKB

Basal production of ROS by granulocytes showed a discrete but significant increase (P<0.05) in cells from type 1 and type 2 diabetic patients in comparison to non-diabetic subjects (tables II and III). Table II shows the modulation by PKA and Akt/PKB inhibitors of the generation of ROS in granulocytes from type 1 and 2 diabetic patients and healthy subjects. The PKA blocker (compound H-89) inhibited ROS production in granulocytes from healthy subjects by 55% (P<0.05), but showed no significant effect (P>0.05) on cells from either type 1 or type 2 diabetic patients. In contrast, the Akt/PKB inhibitor produced no significant effect (P>0.05) on ROS production in granulocytes from non-diabetic controls, but significantly inhibited (P<0.05) the generation of ROS in granulocytes from type 1 and type 2 diabetic patients by 43 and 49%, respectively. These results suggest that the PKA signaling pathway is important for the modulation of ROS production in granulocytes from diabetic patients, whilst Akt/PKB appears to be the main signaling pathway involved in cells from diabetic patients.

Table II

<table>
<thead>
<tr>
<th>Reactive oxygen species</th>
<th>Assay conditions</th>
<th>Non-diabetic subjects (n=30)</th>
<th>Type 1 patients (n=7)</th>
<th>Type 2 patients (n=22)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Granulocytes</td>
<td>2835±125</td>
<td>4304±235</td>
<td>3700±198</td>
<td></td>
</tr>
<tr>
<td>Granulocytes + PKA inhibitor (H89)</td>
<td>1287±94</td>
<td>4365±218</td>
<td>3824±179</td>
<td></td>
</tr>
<tr>
<td>Granulocytes + Akt/PKB inhibitor</td>
<td>2503±106</td>
<td>2454±113</td>
<td>1892±89</td>
<td></td>
</tr>
</tbody>
</table>

* Determined by chemiluminescence assay: values shown are mean measurements of relative light units/min ± standard deviation.

Table III

<table>
<thead>
<tr>
<th>Reactive oxygen species</th>
<th>Assay conditions</th>
<th>Non-diabetic subjects (n=30)</th>
<th>Type 1 patients (n=7)</th>
<th>Type 2 patients (n=22)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Granulocytes</td>
<td>2835±142</td>
<td>4304±217</td>
<td>3700±196</td>
<td></td>
</tr>
<tr>
<td>Granulocytes + dbcAMP</td>
<td>1636±103</td>
<td>9030±423</td>
<td>5882±214</td>
<td></td>
</tr>
<tr>
<td>Granulocytes + dbcAMP + H89</td>
<td>3150±175</td>
<td>7603±358</td>
<td>5050±197</td>
<td></td>
</tr>
<tr>
<td>Granulocytes + dbcAMP + iAkt/PKB</td>
<td>2095±139</td>
<td>1004±96</td>
<td>2804±105</td>
<td></td>
</tr>
</tbody>
</table>

* Mean value significantly different (P<0.05) from that in the assay involving granulocytes and cAMP.

Modulation of the effect of cAMP-elevating agents by inhibitors of PKA and Akt/PKB

Granulocytes from type 1 and type 2 diabetic patients and from healthy controls, were pre-treated with PKA or Akt/PKB inhibitors and then incubated in the presence of a cAMP-elevating agent (dbcAMP) in order to evaluate possible signaling adaptation in cells of diabetic patients. The cAMP-elevating agent (by 43%) ROS production in granulocytes from healthy subjects, but increased production in cells from type 1 and type 2 diabetic patients by 109 and 59%, respectively (table III). Inhibition of the PKA pathway by compound H89 reversed totally the dbcAMP-induced blockade of ROS production in granulocytes from healthy subjects and lead to a 10% increase in ROS compared with non-treated control cells. In contrast, the...
dbcAMP-induced ROS production in granulocytes from type 1 diabetic patients was partially inhibited by the PKA blocker, reducing the initial elevation of 109% to 76% (P<0.05). A similar trend was observed in assays carried out with granulocytes from type 2 diabetic patients, although here the original increase in ROS production of 59% with dbcAMP was reduced to a value of 36% in the presence of the PKA inhibitor. Inhibition of the Akt/PKB pathway reversed the cAMP-induced activation of ROS production in granulocytes from type 1 and type 2 diabetic patients giving rise to a 77% inhibition of production in the former and a 35% inhibition in the latter.

Discussion

cAMP stimulates the production of ROS in granulocytes from both type 1 and type 2 diabetic patients, in contrast to the inhibition observed in cells from healthy subjects, suggesting that ROS generation in diabetic patients could be modulated by intracellular levels of cAMP. We have previously reported that the cAMP-induced inverse ROS metabolic response in granulocytes was PKA-dependent with respect to cells from non-diabetic subjects, and PKA-independent in granulocytes from diabetic patients [7]. This apparent alteration in metabolic response appears to be disease-induced [6], and could be due to chronic hyperglycaemia associated with diabetes.

The involvement of the Akt/PKB signaling pathway in the inverse ROS metabolic response and the possible linking of the production of ROS with the adenylate cyclase system, protein kinases A and B, and the NADPH-oxidase system have been suggested [7]. However, no alteration in the capacity of the NADPH-oxidase system to produce ROS was observed in granulocytes from type 2 diabetic patients [7]. The results of the present study clearly demonstrate that the cAMP-induced ROS response is PKA-dependent in granulocytes from non-diabetic subjects and PKB-dependent in cells from diabetic patients (tables II and III). This change in the signaling pathway may itself be related to chronic hyperglycaemia in diabetes. Some of our patients had retinopathy but not apparent renal failure (table I). We think that the microvascular complications could influence the intensity of ROS generation without affecting the inversion of the granulocyte reactivity mediated by the elevation of cAMP (tables II and III). In the same context, it is well known that metformin has an antioxidant effect modulating the advanced glycation end (AGEs) production and the overproduction of ROS [9]. However, our results showed a significant increase in the basal ROS generation in granulocytes from type 2 diabetic patients (P<0.05) suggesting the absence of interference of this drug (tables II and III).

Mahomed et al [10] suggested that the anti-inflammatory effect of cAMP is a consequence of the enhanced activity of PKA and the subsequent inhibition of production of oxygen species by neutrophils. Indeed, these authors consider that cAMP-dependent PKA is a physiological modulator of superoxide generation by stimulated neutrophils [10]. Our results showing that the intracellular elevation of cAMP blocked the production of ROS by granulocytes from healthy subjects are in agreement with this hypothesis. On the other hand, our experiments with compound H89 (a PKA inhibitor) suggest that whilst the down-regulation is PKA-dependent in healthy subjects it is PKA-independent in diabetic patients. Furthermore, the inhibition and activation of ROS production in the presence of elevated intracellular level of cAMP was reversed following the treatment of cells derived from healthy and diabetic subjects with compound H89 or Akt/PKB inhibitor, respectively (table III). These comparative studies reinforce our suggestion that there is a differential effect of cAMP on ROS generation by granulocytes from healthy subjects compared with those derived from diabetic patients.

For many years the effect of cAMP was believed to be mediated by PKA. However, whilst cAMP/PKA has been shown to be involved in various specific intracellular signaling events, it does not account for all of them. Indeed a number of studies [11-14] have revealed that multiple cAMP pathways exist, but that only some of them are cAMP-dependent. Recently a novel cAMP receptor, known as Epac (exchange protein directly activated by cAMP), was described [15] and this receptor may be important to the greater understanding of cAMP-mediated cell signaling mechanisms. It has been suggested that many of the cAMP functions previously attributed to the cAMP-dependent PKA pathway are, in fact, also Epac-dependent [16]. A Epac family has been suggested [17,18]. In addition to Epac (from now called Epac 1), a second closely related protein has been identified as Epac 2 (cAMP-GEFII), as well as a related protein named Repac (for related to Epac). Tiwari et al. [19] have demonstrated that among circulating hematopoietic cells, peripheral blood B cells express Epac 1 whereas peripheral blood T cells, monocytes and neutrophils do not. However, the effect of Epac on eosinophilic cells has also been demonstrated [20]. Furthermore, whilst cAMP/PKA inhibits PKB stimulation, activation of Epac by cAMP leads to PI-3K-dependent PKB activation. The two intracellular cAMP receptors may, therefore, mediate opposing effects of cAMP on PKB and this may explain the divergent role of cAMP in different cell types [16].

The results obtained in the present study suggest a metabolic adaptation of the generation of ROS in granulocytes from diabetic patients. Thus whilst ROS production is PKA-dependent in healthy subjects, it appears to be preferentially Akt/PKB-dependent in diabetic adapted cells from both type 1 and type 2 diabetic patients. Indeed, the effect of cAMP on ROS production in both type 1 and type 2 diabetic patients was similar (table III), suggesting a metabolic adaptation possibly induced by chronic hyperg-
lycaemia. We suggest that granulocytes in diabetic patients are up-regulated for ROS production by a cAMP/Epac/PI-3K/PKB-pathway, possibly through Epac 2 cAMP-dependent.

It is accepted that the local level of ROS is determined by the balance between its rate of formation by oxidases and auto-oxidation process and its rate of removal via SOD and reaction with various molecules. An increase in the ROS production above a certain threshold (so-called oxidative stress) in inflammatory cells induces damage in adjacent tissue, while low ROS concentration may act as a second messenger. In this context, adhesion of granulocytes (up-regulated for ROS production) to endothelial cells may lead to the development of severe vascular complications. Besides the vascular damage, ROS exert some effects on several signaling pathways involving tyrosine kinases; phosphatases; PKB; PKC; MAPKK; ERK; p38 and JNK. This may suggest that the increase of ROS generation by up-regulated granulocytes induced by cAMP-dependent pathways may cause vascular complications and alteration in the signaling pathways. Increase of ROS generation determines chemical changes in all cellular components leading to DNA and protein modifications and lipid peroxidation. Measurement of biomarkers is a useful tool to evaluate the level of oxidative stress. Despite not using these, they could be important for evaluation of the consequences of increased ROS generation induced by cAMP elevation in cells from diabetic patients.

The signaling pathway involving cAMP, PKA/Epac and/or PKB is very complex and suggests an intricate crosstalk with several other signaling pathways. We still have no satisfactory explanation for the absence of cumulative inhibitory effect of H89 plus cAMP on ROS generation by granulocytes (table III). The balance between PKA/PKB on phosphorylation of p47 phox, a subunit of NADPH-oxidase may be suggested.

Our studies need to be extended with respect to the evaluation of the role of PKC on ROS production and its relation to PKA and PKB in chronic hyperglycaemia. The complex and intricate metabolic signaling pathways associated with ROS production in diabetic patients require further detailed investigation in order to improve our understanding of the implications of this phenomenon on the pathogenesis of diabetes and its possible role in innate immunity.

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References


