Role PKA and p38 MAPK on ROS production in neutrophil age-related: Lack of IL-10 effect in older subjects

Míriam Martins Chaves a,*, Daniela Caldeira Costa c, Bárbara Fonseca de Oliveira a, Marcella Israel Rocha a, José Augusto Nogueira-Machado b

a Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Av. Antônio Carlos 6627, CP 486, 30161-970, Belo Horizonte, Minas Gerais, Brazil
b Santa Casa Hospital of Belo Horizonte, 9 th andar – ala D – Av. Francisco Sales, 1111 – Santa Efigênia, CEP: 30150-221, Belo Horizonte, MG, Brazil
c Departamento de Ciências Biológicas, Instituto de Ciências Exatas e Biológicas, Universidade Federal de Ouro Preto, Moro do Cruzeiro, 35400-000, Ouro Preto, MG, Brazil

1. Introduction

Accumulation of age-related changes age-related is responsible for increasing susceptibility to disease and death (Fulop et al., 2004; Licastro et al., 2005). With aging, there are several changes in immunological and metabolic responses of the receptor-driven function of human neutrophils, such as apoptosis and superoxide anion production (Hayashi et al., 2003; Akgul et al., 2001; Horta et al., 2005; Licastro et al., 2005). We have previously demonstrated that the generation of reactive oxygen species (ROS) is increased with age possibly due to alteration or adaptation, signaling metabolic pathways (Horta et al., 2005; Chaves et al., 1998, 2000, 2002, 2007, 2008). It is believed that ROS production is associated with an inflammatory process; which involve a complex network of cytokines and cellular interactions (Fulop and Seres, 1994). Pro- and anti-inflammatory cytokines have a pivotal role in inducing and modulating such inflammatory process (Bruce et al., 2003; Grandjean-Laquerriere et al., 2003). IL-10 effect depends on PKA and cAMP, and the lack of effect on the elderly is p38 MAPK dependent.
2. Subjects and methods

2.1. Reagents

Interleukin 10 (IL-10) was purchased from Sigma Co.; PKA inhibitor (H89), Akt/ PKB inhibitor (iAkt), p38 MAPK kinase inhibitor (PD169316) and NADPH inhibitor (DPI) were purchased from Calbiochem.

2.2. Subjects

The Ethical Committees at the Federal University of Minas Gerais and the Santa Casa de Misericórdia of Belo Horizonte—a nonprofit hospital, have approved these studies. Detailed medical history, physical examination and laboratory data for each subject were carried out 4 weeks before entering the study. According to a senior study protocol for the elderly immune function, Lichter et al. (1984), 43 people were excluded because they showed conditions incompatible with admission criteria (i.e., smokers, infections, inflammation, malignancy, lymphoproliferative disorders, arteriosclerosis, cardiac insufficiency, hypertension, dementia, pregnancy, alcoholism and drug abuse) and/or were taking known drugs that directly influence the immune function. The subjects were divided into five age groups as follows: elderly group: (age 70–80) and (age 60–69) with 20 individuals (10 males and 10 females) and 25 individuals (12 males and 13 females), respectively; middle adult group (age 50–59) and (age 40–49) with 21 individuals (10 males and 11 females) and 26 individuals (13 males and 12 females), respectively and younger adult group (age 20–39) included 50 individuals (27 males and 23 females). These groups were selected by Dr. Maria Regina Calsolari and these individuals lived in Belo Horizonte, Minas Gerais, Brazil.

2.3. Cell separation

The neutrophils were purified from 10 ml of heparinized venous blood. The cells were isolated by Ficoll–Hypaque gradient according to Bicalho et al. (1981), with slight modifications. Briefly, the neutrophils were separated by cell centrifugation over two gradients with density of 1.08 and 1.13 in order to obtain the simultaneous separation of mononuclear cells and neutrophil, respectively. The purity of the neutrophil preparation of was of 95–100%. The viability of each sample was always greater than 98% as determined by the Trypan Blue exclusion test.

2.4. Gamma interferon (IFN-γ) production

A gamma interferon-rich supernatant was produced by incubation of peripheral blood mononuclear cells (PBMC) [1 x 10^7/ml RPMI-1640 (cell culture medium)] with Concanavalin A (Con A) at 37 °C, in 5% CO₂ for 30 min. After incubation, the cells were transferred to tubes and centrifuged at 400 × g for 30 min at room temperature. The pellet obtained was resuspended in RPMI-1640 medium and a new incubation was performed at 37 °C in 5% CO₂ for 18 h. After the last incubation, the cells were centrifuged (400 g for 30 min) and the supernatant-cell free and interferon-gamma (IFN-γ)-rich was collected and tested. A pool of supernatant was performed from several cell cultures. The best quantity of supernatant IFN-γ-rich was achieved using a curve dose–response. The quantities tested for IFN-γ were (100, 200 and 300 μl). The quantity of 200 μl (100 μg/ml) corresponds to a higher activation of ROS generation in conjunction with a lower percentage of dead cells (>89%). The pre-incubation of the supernatant interferon-gamma rich (200 μl) together with monoclonal antibody anti-IFN-γ abolish this stimulatory effect on neutrophil from age subjects.

2.5. ROS production

The quantitative basal ROS determination was performed in a luminol-dependent chemiluminescence assay by the incubation of neutrophil (1 x 10^6 cells/100 μl PBS phosphate buffered saline) with luminol for 40 min. A luminol [Sigma Co.] stock solution was made by dissolving 1.77 mg of luminol in 1.0 ml dimethyl sulphoxide (DMSO) to render a concentration of 10^-3 M, which was diluted to 10^-8 M in PBS (pH 7.3) before put into use. Neutrophil was pre-incubated with luminol in test tubes for 10 min. Subsequently, it was incubated with 10 μl of IL-10 (10 μg/ml) and 200 μl of IFN-γ (100 μg/ml). In the remaining experiments, neutrophil was incubated with both H89 (PKA inhibitor) (1 μM), Akt/PKB inhibitor (iAkt) (1 μM), p38 MAPkinase inhibitor (PD169316) (10 μM) and NADPH inhibitor (DPI) (10 μM) for 30 min and then neutrophil was incubated with 10 μl of IL-10 (10 μg/ml) for another 30 min period. Each unsealed luminescence tube received 0.1 ml of luminol and the final volume was adjusted to 700 μl. The chemiluminescence measurements were performed in a luminometer 1250-101 (Lumat, LB 9501, EG&G Berthold, Germany). The experiments were performed in duplicate at 37 °C. The chemiluminescence was recorded during 40 min; it was time enough for observing the peak. The results were expressed in relative light units/min (RLU/min). The control experiments were carried out simultaneously.

2.6. Dose–response curve

A dose–response curve was obtained by adding increasing concentrations of H89 (PKA inhibitor), Akt (Akt/PKB inhibitor), PD169316 (p38 MAPKinase inhibitor) and DPI (NADPH inhibitor). The cell viability measured was 93% (1 μM), 94% (10 μM), 95% (30 μM) for both inhibitors. However the best inhibitory concentrations were: (1 μM) H89 and iAkt and (10 μM) PD169316 and DPI.

2.7. Statistical analysis

Statistical analyses were performed using Mann–Whitney non-parametric test; non-parametric Kruskal–Wallis with Dunn’s post hoc test.

3. Results and discussion

Cells are continuously submitted to the oxidative damages caused by reactive oxygen species (ROS), which are produced during a normal aerobic metabolism of mitochondria (Cassatella et al., 1991). An attack of ROS on cellular components has been considered as the main cause of senescence, cellular death and related phenomenon. The values represent the mean ± S.E. Reactive oxygen species (ROS) generation was expressed as relative light units (RLU/min) during 40 min reaction. G = granulocytes; IL-10 (Interleukin 10); H89 = (PKA inhibitor); iAkt/ PKB = (PKB inhibitor); PD169316 = (p38 MAPK inhibitor) PBS = phosphate buffered saline. *Significant when compared with respectively controls by Mann–Whitney test. Age groups (years old) = (50–59), (60–69) and (70–80) differ from (20–39) and (40–49) at p < 0.008 (***) by Kruskal–Wallis test for multiple comparisons.

Fig. 1. Differential signaling of interleukin 10 (IL-10) on ROS generation: an age-related phenomenon. The values represent the mean ± S.E. Reactive oxygen species (ROS) generation was expressed as relative light units (RLU/min) during 40 min reaction. G = granulocytes; IL-10 (Interleukin 10); H89 = (PKA inhibitor); iAkt/ PKB = (PKB inhibitor); PD169316 = (p38 MAPK inhibitor) PBS = phosphate buffered saline. *Significant when compared with respectively controls by Mann–Whitney test. Age groups (years old) = (50–59), (60–69) and (70–80) differ from (20–39) and (40–49) at p < 0.008 (***) by Kruskal–Wallis test for multiple comparisons.
inflammation (DeLeo and Quinn, 1996). It is known that the IL-10 is an anti-inflammatory interleukin produced during inflammatory processes in vivo. Thus, IL-10 has been identified as an important regulator of immune, inflammatory processes and of aging.

For understanding the biochemical mechanisms involved in IL-10 inhibition of ROS production, we have used selective inhibitors such as H89 (PKA inhibitor), iAkt (Akt/PKB inhibitor) and PD169316 (p38 MAPK inhibitor). The results are showed in Fig. 1. The age-related ROS production increased. The ROS values expressed as RLU/min ranged from 2.156 ± 39.0 (for the 20–39-year-old group) up to 6.298 ± 587 (for the 70–79-year-old group) controlled with neutrophil and PBS (Fig. 1, panel A–C). The metabolic route PCA–but not PKB or p38 MAPK–dependent on down-regulated ROS production at ages 20–49 (Fig. 1, panels A–C). PKA is considered as a receptor of cAMP (Zheng and Martinez, 1999; Dodge and Samborn, 1998; Acin-Perez et al., 2009). Some reports have demonstrated the association between IL-10 and the level of cAMP (Cassatella, 1998; Dumont et al., 1989). However, for the age group 50 years old and more, the p38 MAPK inhibitor (PD169316), but not H89, was able to decrease the ROS production (Fig. 1, panel C). It is known that aging affects the basal level of signaling when relying to stress, as well as the pathways of JNK and p38 MAPK (Eom and Choi, 2009). As for polymorphonuclear leukocytes, the importance of p38 MAPK was recently reported as a probable responsible for signaling the ion superoxide’s production (Zhang and Yang, 2006; Gong et al., 2008; Han et al., 2009). IL-10 inhibits ROS production up to age 50, but has no effect on older subjects (Fig. 1, panel C). Thus, we have suggested that ROS modulation may use the PKA pathway for IL-10 in younger subjects; and the modulation in older subjects depends on P38 MAPK. (Fig. 1, panels A–C). It may also be suggested a pro-inflammatory state to older subjects due to an alteration of metabolic signaling response to IL-10 as for the ROS generation.

Certainly, it has an important effect, because IFN-γ (Table 1) is a pro-inflammatory cytokine (Yoon et al., 1997; Weyand et al., 2003), which is able to activate ROS production. The activation IFN-γ-mediated was observed in all age groups, while the inhibition induced by IL-10 (anti-inflammatory cytokine) was decreasing in an age-related manner (66% for ages 20–39; 52% for ages 40–49; 16% for 50–59; 5% for 60–69; and 11% for those aged 70–79). The activation of ROS generation in IFN-γ-stimulated neutrophils was down-regulated by the IL-10 and it has been suggested that the effect of IL-10 involves CAMP and PKA.

Several works have demonstrated that the generation of superoxide anion by the complex NADPH oxidase is accompanied by an extensive phosphorylation of its 47-kDa protein component, p47phox, a major cytosolic component of this oxidase (Dang et al., 2001; Dekker et al., 2000; Karlsson et al., 2000). Our data with DPI suggest that the inhibition of ROS production mediated by DPI (NADPH oxidase inhibitor) or by IL-10 involves different metabolic routes (Table 2). The results with DPI suggest that the main source of ROS production in neutrophil is the NADPH-oxidase system (Arruda and Barja-Fidalgo, 2009; El-Benna et al., 2009; Kim and Cha, 2009). DPI inhibited ROS production in a similar way for all age groups. It was not observed with IL-10 however, which appears to have a differential effect on neutrophil in aging dependence (Table 2).

This differential effect of age-related IL-10 may suggest adaptation or even alteration in signaling pathways involving ROS production in young people as compared with old people. The impact of these findings is extremely important for the understanding of immunoregulatory mechanisms of aging and the loss of IL-10 inhibitory function on ROS generation could be associated with the increase of ROS-induced cellular damages.

In conclusion, the present results suggest the importance of an age-related imbalance between signaling pathways involving PKA, p38 MAPK in the senescence pro-inflammatory profile.

Acknowledgements

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References


Table 1

Lack of IL-10 effect on stimulatory effect of IFN-γ on neutrophil from older person.

<table>
<thead>
<tr>
<th>Age group (years)</th>
<th>N</th>
<th>Chemiluminescence ± S.E. in RLU/min</th>
<th>G + PBS</th>
<th>G + IFN-γ</th>
<th>G + IL-10</th>
<th>G + IFN-γ + IL-10</th>
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<tr>
<td>20–39</td>
<td>35</td>
<td>2196 ± 305</td>
<td>29.9 ± 3.4</td>
<td>31.0 ± 3.6</td>
<td></td>
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<tr>
<td>40–49</td>
<td>32</td>
<td>3921 ± 361</td>
<td>28.0 ± 4.5</td>
<td>35.5 ± 2.0</td>
<td></td>
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<tr>
<td>50–59</td>
<td>25</td>
<td>4654 ± 401</td>
<td>31.0 ± 4.0</td>
<td>39.4 ± 3.2</td>
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<tr>
<td>60–69</td>
<td>20</td>
<td>5129 ± 475</td>
<td>32.3 ± 3.0</td>
<td>38.4 ± 2.9</td>
<td></td>
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<tr>
<td>70–80</td>
<td>18</td>
<td>6232 ± 598</td>
<td>37.0 ± 5.0</td>
<td>39.0 ± 3.2</td>
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The values represent the mean ± S.E. Reactive oxygen species (ROS) generation was expressed as relative light units (RLU/min) during 40 min reaction. G = granulocytes; DPI = (NADPH-oxidase inhibitor); PBS = phosphate buffered saline. A, B, C, D, E differ from the respectively control by Mann–Whitney test p < 0.001.

Table 2

Lack of IL-10 effect no interferes in the NAPH-oxidase activity in age process.

<table>
<thead>
<tr>
<th>Age group (years)</th>
<th>N</th>
<th>Chemiluminescence ± S.E. in RLU/min</th>
<th>G + PBS</th>
<th>G + DPI</th>
<th>G + DPI + IL-10</th>
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