Enhanced ROS-Generation in Lymphocytes from Alzheimer's Patients

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Original Paper

Introduction: Reactive oxygen species (ROS) have been implicated in neurodegeneration and seem to be involved in the physiology and pathophysiology of several diseases, including normal aging and Alzheimer's disease (AD). Enhanced ROS production in aging or AD is not restricted to the brain, but can also been seen in several peripheral tissues. The objective of the present study was to evaluate whether the mechanisms involved in the generation of oxidative stress in normal senescence and Alzheimer's disease are identical or not. **Methods:** We analysed intracellular basal levels of ROS in lymphocytes from AD patients and healthy young and aged not-demented subjects as well as ROS levels following stimulation with d-ribose and staurosporine in all three groups. ROS levels were measured by flow cytometry using the intracellular fluorescence dye dihydrorhodamine123 (DHR123). **Results:** Our study shows that AD lymphocytes have increased basal levels of ROS, low susceptibility to ROS stimulation by 2-deoxy-D-ribose (dRib) and an increased response to staurosporine when compared with age-matched controls. **Discussion:** The data suggest that the defect(s) responsible for enhanced ROS production in AD may involve different or additional biological pathways than those involved in enhanced ROS generation during aging.

Introduction

Several hypotheses have been proposed as molecular basis explaining the enhanced neurodegeneration occurring during normal aging and Alzheimer's disease (AD). One of the most compelling is the role of free radical-induced oxidative stress in these disorders [1,16,17,22,24,25]. Enhanced oxidative stress during aging and AD is not restricted to the brain but also present in peripheral cells like fibroblasts [11] or lymphocytes [6,22,23]. Several oxidative stress-related changes in lymphocytes have been observed related to aging as for example diminished concentrations of antioxidants like α-tocopherol, ascorbat and glutathione [15] or age-associated changes in mitochondrial function including a decrease of several complexes of the respiratory chain and increased levels of oxidized mitochondrial DNA [6,12].

Some of these changes ave additionally elevated in peripheral cells from patients with Alzheimer's disease (AD) like decreased glutathione (GSH) content [3] increased levels superoxide dismutase (MnSOD) mRNA [5] or increased lipid peroxidation [4]. Some findings indicate increased oxidative damage in lymphocytes of Alzheimer's disease patients [18–21]. Moreover, similar changes may be induced by AD specific mutations of APP and PS1 genes. Gibson et al. demonstrated that fibroblasts bearing the AD Presenilin-1 246 Ala→Glu mutation have altered means of hand-

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ling oxidative stress [10]. Comparable findings were reported for lymphocytes of mice bearing PS1 mutations [9].

In order to investigate whether similar or divergent mechanisms of ROS generation are relevant for aging and sporadic AD, we investigated levels of ROS in young and aged human lymphocytes as well as in lymphocytes from AD-patients. In contrast to measurements of plasma lipid peroxidation products or the activities of antioxidant enzymes in blood cells, determination of intracellular ROS (reactive oxygen species) may directly reflect oxidative stress inside the cell.

Materials and Methods

Subjects

For the study comparing ROS levels in young and aged humans, we used fresh blood samples from n = 47 healthy subjects (21 women and 26 men) between 20 and 86 years. There were 10 women and 13 men in the aged group (n = 23; aged over 60 years, mean age 69.4 ± 6.8 years) and 11 women and 13 men in the group of young controls (n = 24; age under 35 years, mean age 27.38 ± 3.81 years).

For the determination of ROS levels in lymphocytes from aged not-demented controls and patients with Alzheimer's disease, blood samples were taken from n = 53 subjects (33 women and 20 men) between 57 and 91 years. There were 14 women and 11 men in the aged group (n = 25; 60–85 years, mean age 71.7 ± 7.5 years) and 19 women and 9 men in the Alzheimer's group (n = 28; 57–91 years, mean age 72.7 ± 10.0 years). Dementia was diagnosed according to ICD-10. Diagnosis of Alzheimer's disease was achieved following the guidelines of the National Institute of Neurological and Communicative Disorders and Stroke–Alzheimer's Disease and Related Disorders (NINCDS-ADRDA criteria) task force. The majority of cases exhibited mild to moderate dementia.

Subjects with pathological distribution of lymphocyte subpopulations in flow-cytometric analysis were rejected from the study. Most of the patients were taking drugs against cardiovascular disorders. None of the control patients received psychotropic medication, drugs with known effects on the immune system, or antioxidative drugs. The study was approved by the responsible Ethical Committee and written informed consent from all subjects was received, where appropriate, from their caregivers.

Cells

Peripheral blood lymphocytes were prepared from whole fresh blood by separating on Ficoll (Biochrom KG, Berlin, Germany) as previously described [8]. Cells were investigated after the incubation with two different stimuli or with buffer as control.

Measurement of oxidative stress

The determination of ROS was performed according to a previously described procedure [22]. In brief, lymphocytes were incubated with the dye dihydrorhodamine123 (DHR123; 10 μmol/l; Molecular Probes, Netherlands) for 15 minutes at 37 °C. Then, the conversion of DHR123 to its fluorescent derivative rhodamine123 was detected by FACS analysis. Probes were stimulated either for 16 hours with 50 mM 2-deoxy-D-ribose (dRib) or 500 nM staurosporine.

Results

Altered generation of ROS in aging

Oxidative stress was induced with either 2-deoxy-D-ribose (dRib) or the apoptotic agent staurosporine. Cells were incubated for 16 hours at 37 °C after the addition of one of the stimuli mentioned or of buffer as control. The oxidative-sensitive fluorescent dye DHR123 was used to detect presence of ROS in human lymphocytes. Basal levels of ROS (expressed as mean fluorescence intensity in arbitrary units) accumulated significantly in aged human lymphocytes (Fig. **1A**; $^{*}p < 0.05$; n = 22–24).

Incubation with 2-deoxy-D-ribose (dRib), a reducing sugar that induces apoptosis in quiescent human lymphocytes through oxidative mechanisms [13], led to an age-related increase in ROS levels (Fig. **1B**; $^{*}p < 0.05$; n = 21–23). In addition, treatment with the apoptotic stimuli staurosporine generates increased levels of ROS without any age-related differences (Fig. **1C**; n = 16).

Altered generation of ROS in Alzheimer's disease

After a period of 16 hours in vitro incubation, basal levels of ROS were significantly increased in lymphocytes of Alzheimer's patients (Fig. **2A**; $^{**}p < 0.01$; n = 23–25). The incubation with dRib (50 mM) led to an increase in DHR123 fluorescence without any Alzheimer-related difference (Fig. **2B**; n = 24–28).

In contrast, treatment with staurosporine (500 nM) evoked a significantly larger increase in ROS levels in lymphocytes from Alzheimer's patients than from healthy controls (Fig. **2C**; $^{*}p < 0.05$; n = 23–25).

Discussion

Only few observations indicating enhanced oxidative stress have been reported for lymphocytes from AD patients, such as de-

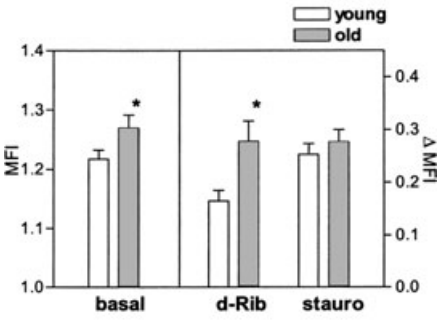

Fig. **1** Levels of ROS in lymphocytes from young and old subjects measured with the fluorescent dye DHR123. **A)** Basal levels of ROS in human lymphocytes. Significantly higher levels could be determined in aging (old, n = 22; versus young, n = 24; $^{*}p < 0.05$). **B)** Induced ROS levels over control in lymphocytes cultured for 16 h with dRib. There was an age-related increase in DHR123 fluorescence (Δ increase over baseline; old, n = 23; versus young, n = 21; $^{*}p < 0.05$). **C)** Induced ROS levels over control in lymphocytes cultured for 16 h with staurosporine. There was an increase in ROS without any age-related differences (Δ increase over baseline; old, n = 16; versus young, n = 16).

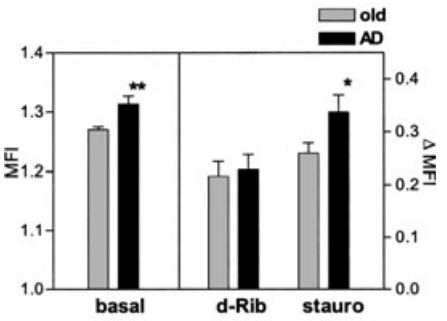

Fig. **2** Levels of ROS in lymphocytes from old subjects and patients with Alzheimer's disease (AD) measured with the fluorescent dye DHR123. **A)** Basal levels of ROS in human lymphocytes. Significantly higher levels could be determined in AD (AD, $n = 23$; versus old, $n = 25$; $^*p < 0.01$). **B)** Induced ROS levels over control in lymphocytes cultured for 16 h with dRib. There was an increase in DHR123 fluorescence without any age-related differences (Δ increase over baseline; AD, $n = 28$; versus old, $n = 24$). **C)** Induced ROS levels over control in lymphocytes cultured for 16 h with staurosporine. There was an age-related increase in ROS (Δ increase over baseline; AD, $n = 23$; versus old, $n = 25$; $^*p < 0.05$).

creased glutathione (GSH) content [3], altered calcium dynamics [7] as well as enhanced oxidative DNA damage [18]. The experiments in the current study demonstrate that cells from AD patients handle oxidative stress differently than aged-matched controls when incubated with two different stimuli of ROS.

The reducing sugar 2-deoxy-D-ribose (dRib) has been shown to induce apoptosis in a variety of cells. Apoptosis seems to be generated by free radicals and mitochondrial dysfunction. A time-dependent marked increase of depolarised mitochondria was observed in cultures exposed to dRib, possibly occurring as a consequence of both, production of radicals and/or significant glutathione depletion. We have already previously shown that treatment with dRib evokes increased levels of ROS in human lymphocytes [22]. Other authors report that dRib treatment induced complete depletion of reduced glutathione (GSH), a key molecule for anti-oxidant protection [13]. They hypothesize that dRib, by lowering the intracellular levels of GSH until complete depletion leads to an oxidative imbalance inside cells. There are also findings indicating disturbances of cytoskeletal integrity and cell adhesion [13].

Staurosporine is widely employed as an inducer of apoptosis in many cell types. The intracellular cascades that mediate staurosporine-induced apoptosis are largely unknown. ROS have been implicated in staurosporine-induced changes of mitochondrial membrane potential, cell cycle arrest and the activation of caspase-3 by the accumulation of cytochrome c in the Cytosol [2]. It has been shown that mitochondrial respiratory chain is the principal source of ROS generation in staurosporine-induced apoptosis [2]. Moreover, staurosporine induces an early increase in cytosolic calcium followed by delayed increase of mitochondrial calcium. Mitochondrial ROS accumulation is also described [14].

In our study, basal ROS levels were increased in aged subjects and were further increased in lymphocytes from AD patients. These results confirm the above mentioned observations indicating additionally altered metabolism of free radicals also in cells from AD patients.

Stimulation of lymphocytes from young and old donors showed no age-related difference in the extent of ROS-generation when incubated with staurosporine, however, treatment with dRib evokes significant higher ROS generation in cells from aged subjects than in young controls. Since dRib is particularly effective in depleting intracellular levels of GSH, the already decreased intracellular levels of glutathione in lymphocytes from aged relative to young subjects may be an explanation for the latter findings [15].

Lymphocytes from AD patients show no differences in ROS generation compared to age-matched controls when incubated with dRib, suggesting that levels of glutathione are not further decreased in cells from AD patients. On the other hand, cells from AD patients but not from aged humans are more sensitive to ROS generation induced by staurosporine. The exact mechanism remains unknown. We can only speculate that the above mentioned alterations of mitochondrial function in AD lymphocytes, might contribute to the enhanced sensitivity to the staurosporine [23 – 25].

The main finding of the present study is that two different stimuli which trigger the generation of free radicals in human lymphocytes via different mechanisms evoke different answers in cells from aged subjects and patients with Alzheimer's disease. We can conclude that the mechanisms involved in the generation of free radicals are different in the pathology of Alzheimer's disease and in senescence.

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