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Research paper

Involvement of acetylcholinesterase and protein kinase C in the protective effect of caffeine against β-amyloid-induced alterations in red blood cells

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ABSTRACT

It is well known the role of oxidative stress in the pathophysiology of Alzheimer's disease (AD) and of other neurodegenerative pathologies. We have previously documented that Amyloid beta peptide (1–42) (Abeta) dependent-oxidative modifications affect red blood cell (RBC) morphology and function. Experimental studies show that caffeine (CF) consumption is inversely correlated with AD. In this study, we investigated the role played by RBC in the protective mechanism elicited by CF against Abeta mediated toxicity. PS exposure levels by FACS analysis, as well as protein band 3 functionality analysis, indicated that CF at 100 μM protected against Abeta-mediated membrane alterations, which are known to occur in AD. Moreover, CF counteracts inhibition of ATP release from RBC by Abeta, restoring its ability to modulate vasodilation. Concurrently, analysis of protein kinase C (PKC) and caspase 3 activities, responsible for cytoskeleton alterations, revealed that unlike to caspase 3, PKCα activation induced by Abeta was fully abolished by CF through a mechanism involving Acetylcholinesterase (AChE), located on external face of RBC plasma membrane. These results provide support for the hypothesis concerning the protective role of CF in AD patients could include also a peripheral mechanism involving RBC.

1. Introduction

Caffeine (CF) is a xanthine derivative that acts in the body's cell by several mechanism of action on a wide range of molecular targets [1]. Recently, CF has aroused scientific interest because of its potentiality as an antioxidant compound able to protect against oxidative stress [2] and Alzheimer's disease (AD) like pathology [3]. With respect to red blood cells (RBCs), particularly remarkable are some studies related to the potential antioxidant properties of the CF [4–6]. In particular, previous studies report that CF influences glutathione S-transferase activity, and inhibits RBC membrane derangement and suicidal cell death [4–6]. Furthermore, some of us [5], recently reported a strong scavenger capacity of CF toward hydroxyl radical, already evident at low doses of the alkaloid. In particular, the antioxidant activity of CF is so high that it persists even in RBC incubated with a strong oxidizing agent such as tert-butyl hydroperoxide (t-BHT), well known radical oxygen species (ROS) generator. Abeta toxic effects lead to an increase in oxidative stress [7–9]. Abeta peptide was found in blood at nanomolar concentrations and it is abundantly produced by platelets [10,11]. RBCs come into contact with Abeta at the level of the luminal surface of brain capillaries [12] and they seem to interact with monomeric Abeta [13]. Vasculature alterations are closely linked with AD [14] and the decreasing in RBC deformability is associated with the appearance of vascular alterations that characterize AD [15,16].

Abbreviation: Abeta, Amyloid beta peptide (1–42); AD, Alzheimer's disease; CF, caffeine; AChE, Acetylcholinesterase; PKC, protein kinase C; eNOS, endothelial nitric oxide synthase; RBC, red blood cell; PS, Phosphatidylserine.

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Previous studies [6,17–22], show that Abeta affects RBCs metabolism and induces RBCs death. In our previous studies we outlined a caspase 3 activation after Abeta exposure [23], leading to band 3 cleavage in its cytoplasmic domain [24]. In a very recent manuscript, some of us demonstrated that in RBC, protein kinase C (PKC) in consideration of its known role in RBC [25,26], could play a key role in Abeta-induced oxidative imbalance [27].

Acetylcholinesterase (AChE) located on the external face of RBC plasma membrane, could represent a favorite target for Abeta [19].

Abeta toxic effects are mediated by AChE [28,29] located on RBC membrane. AChE is highly expressed in RBCs than other blood cells [30]. On this basis, we have asked whether neuro-protective action of CF against AD, could involve also a peripheral mechanism including RBC, that is known to contribute to the pathophysiology of AD.

2. Materials and methods

2.1. Chemicals

Abeta, with a certified purity of >98%, was obtained from Peptide Specialty Laboratories GmbH (Heidelberg, Germany). Peptides lyophilized powder was stored at −20 °C. The 1,1,1,3,3,3 hexafluoro-isopropanol (TCI America, Portland, OR, USA) was used to treat peptides, before use, to exclude aggregates formation. Abeta was stored as dry films as described previously [31]. Other reagents were purchased from Sigma Chemical Co. (St. Louis, MO; USA).

2.2. Ethics statement

Blood samples were obtained from healthy individuals of 30 ± 5 years old, abstained from drug treatments and from CF consumption for at least one week prior to sample collection. No blood samples were obtained from regular blood donors.

The blood samples collection was performed according Helsinki Declaration.

2.3. Preparation of RBCs and incubation conditions

Whole blood (3 ml) was collected with citrate as an anticoagulant, and washed three times with an iso-osmotic NaCl solution. Plasma separation was obtained by centrifuging at low speed (800 × g for 5 min), in order to avoid any RBCs morphological alteration induced by mechanical stress. A density gradient centrifugation with Ficoll was used to isolate mature RBCs [32].

After washing step, 45 μl of packed RBCs were re-suspended, in a final volume of 1.5 ml (haematocrit 3%) of the incubation buffer (35 mM Na2SO4, 90 mM NaCl, 25 mM HEPES [N-(2-hydroxyethyl)-piperazine-N1-2-ethanesulfonic acid], 1.5 mM MgCl2, glucose 5 mM).

Suspensions obtained were incubated, in the above buffer for 12 h at room temperature with or without 5 μM of Abeta, as previously reported [33] and CF 100 μM [5]. A speed centrifugation of 500 × g was used to sediment RBCs excluding cell lysis.

After incubation, Met-hemoglobin (met-Hb) and haemolysis levels were determined.

2.4. AChE enzyme assay

After Abeta treatment, AChE activity was measured as previously reported [34].

2.5. FACS analysis to measure PS exposure

Following the method described by Andree et al. [35], the fluorescence activated cell sorting (FACS) analysis was performed. After RBC exposition to different experimental conditions, they were washed with a buffer containing 0.14 M NaCl, 0.01 M HEPES-NaOH (pH 7.4), and 2.5 mM CaCl2 (annexin-binding buffer). The successive step was RBC staining with Annexin-V-Fluos and annexin buffer.

Samples were diluted 1:5 in annexin-binding buffer subsequently to 10 min incubation in the dark. This samples was used to perform flow cytometric analysis.

The annexin fluorescence intensity was measured in fluorescence channel FL-1 through the forward scatter analysis. The excitation and emission wavelengths were 488 nm and 530 nm respectively.

2.6. PKC assay

After different treatments, a 1100 × g centrifugation at 4 °C for 5 min was used to collect RBCs as reported by Klarl et al. [36] and washed with 1 ml PBS.

Subsequently to the washing step, 150 μl of a buffer with 20 mM Tris HCl (pH 7.4), 1 mM sodium orthovanadate, 5 mM EGTA, 1% Triton X-100 plus a protease inhibitors solution (10 μg/ml pepstatin A, 10 μg/ml leupeptin, 5 μg/ml aprotinin, and 0.1 mM PMSF) (Roche Diagnostics), was used to lyse RBCs.

The lysates were incubated on ice for 30 min and then were pelleted for 15 min at 22,000 × g at 4 °C. The Bradford method (Bio-Rad, Munich, Germany) was used to determine the supernatant protein concentration with BSA (Sigma) as a standard.

The StressXpress PKC Kinase Activity Assay Kit from Stressgen (Biomol- Hamburg, Germany) has allowed us to determine PKC activity in RBCs extracts.

The analysis was performed according the methods previously described by Klarl et al. [36].

2.7. Band 3 anion exchanger activity determination

After washing RBCs were incubated at 25 °C, in the above incubation buffer, with or without.

CF (100 μM) or Abeta (5 μM) and with Abeta (5 μM) plus CF (100 μM). Sulphate influx was assayed by using the method as previously reported [37].

The following equation was used to calculate sulphate concentration as a function of the incubation time: $c(t) = c_{\infty}(1 - e^{-}kt)$. In this equation c(t) is the sulphate concentration at time t, c_{∞} is the intracellular sulphate concentration at equilibrium and k is the sulphate influx rate constant.

2.8. Measurement of extracellular ATP

ATP levels were measured as previously reported [38]. The crude firefly tail extract added to synthetic d-luciferin, was used to improve test sensitivity. In a cuvette with 100 μl of crude firefly tail extract (10 mg/ml distilled water, FLE 250; Sigma–Aldrich) plus 100 μl of synthetic D-luciferin (50 mg/100 ml distilled water; Sigma–Aldrich) was added 200 μl of RBC suspension.

A 1251 luminometer BioOrbit was used to detect the light emitted. During each experiment a standard curve was obtained. ATP levels were measured as above described after dilution of TCA to 0.01%, to avoid assay interference. obtained values were normalized to RBC ATP concentration.

2.9. Caspase 3 assay

Caspase activity was performed as previously reported [23]. Briefly, after different treatments, a 3000 × g centrifugation for