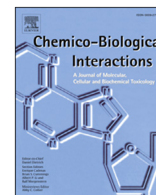
Chemico-Biological Interactions 270 (2017) 24–32

Contents lists available at ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint



Carbamylation and oxidation of proteins lead to apoptotic death of lymphocytes

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ARTICLE INFO

Article history:
Received 26 January 2017
Received in revised form
24 March 2017
Accepted 7 April 2017
Available online 8 April 2017

Keywords:
Carbamylation
Oxidation
Lymphocytes
Apoptosis
Caspases
Mitochondrial potential

ABSTRACT

The apoptotic/necrotic changes in isolated human peripheral blood mononuclear cells (MNCs) subjected to hydrogen peroxide (H_2O_2), cyanate (NaOCN) and their combination were examined. The mitochondrial potential (ΔΨm), the activities of caspases (−2, −3, −6, −8 and −9) and the level of carbonyls and amino groups in proteins were determined and DNA fragmentation. Apoptotic or necrotic cells were identified by fluorescence microscopy using double staining with Hoechst 33258/propidium iodide.

Treatment of MNCs with NaOCN (1 mmol/L and 2 mmol/L), alone and in combination with H_2O_2 (100 μmol/L), led to a significant decrease in the content of amine groups and a significant increase in the carbonyl level of MNCs in comparison with the control. Measurements taken at three time points (30, 60 and 150 min) showed a significant decrease in ΔΨm in MNCs incubated with H_2O_2, cyanate and their combination. The highest decrease in ΔΨm was observed after 150 min, when a combination of NaOCN and H_2O_2 was applied.

We observed significant increases in the activities of caspases-2 and -3 in cells exposed to H_2O_2 and the combination of NaOCN and H_2O_2. An increase in caspase-2 but not in caspase-3 activity was noted in cells incubated with cyanate. A significant increase in caspase-9 activity in MNCs was observed in all arrangements of tested compounds in comparison with the control.

In H_2O_2-treated cells, a higher level of necrotic cells was noted in comparison to apoptotic cells, whereas carbamylation led mainly to apoptotic cell death. The combination of cyanate and H_2O_2 increased the population of necrotic cells.

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1Introduction

Carbamylation (carbamoylation) of biological materials is a post-translational modification resulting from nonenzymatic reactions between isocyanic acid and free functional groups of macromolecules. This process plays a key role in the pathogenesis of several diseases, such as chronic renal failure, cardiovascular disease, cataract and rheumatoid arthritis [45]. It is known that isocyanate is in equilibrium with cyanate, which is in another equilibrium with urea. Isocyanate leads to carbamylation of peptides, proteins, lipids, enzymes and other biological molecules [22,52]. In the plasma of healthy subjects, low concentrations of cyanate can be detected, but they are significantly elevated in patients with renal dysfunction. In patients with chronic renal failure, the concentration of urea in the blood is 5–7 times higher than in healthy individuals [28,44]. Higher levels of urea in the plasma of chronic renal failure (CRF) patients lead to an increase in the concentration of cyanate and its reactive form, isocyanate. Chronic renal failure is accompanied by modifications of biological materials, which are caused by both processes – carbamylation and oxidation. Carbamylation is a nonenzymatic process, but in inflammation under physiologic conditions, it can be catalysed by haeme-dependent peroxidase, neutrophil myeloperoxidase (MPO) and eosinophil peroxidase (EPO) [54,55]. MPO and EPO catalyse the oxidation of halides to hypochlorous acid and hypobromous acid, respectively, both strong oxidized and halogenated agents [14,29]. Myeloperoxidase may also catalyse the oxidation of thiocyanate/isothiocyanate in the presence of hydrogen peroxide, producing cyanate/isocyanate at sites of inflammation [53].

In addition, haemodialysis patients are particularly exposed to oxidative stress [18]. Haemodialysis induces the activation of neutrophils and the release of reactive oxygen species (ROS), such as

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http://dx.doi.org/10.1016/j.cbi.2017.04.007
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superoxide radical, hydroxyl radical, hydrogen peroxide and hypochlorous acid [11,12,16]. In haemodialyzed patients, the oxidation process is intensified due to the release of haemoglobin from red blood cell damage by rotary blood pumps [39,42]. This haemoprotein can be oxidized to the ferryl state (Fe IV=O), by hydrogen peroxide and/or hydroperoxide, giving another strong oxidizing agent [21]. In addition, haemoproteins can be released from the iron-catalyst of free radical reactions [27]. Free iron may also be released from ferritin by nitric oxide, because the trigger production of this species was noted in haemodialyzed CRF patients [40,1]. There is evidence, in the form of tyrosine nitration in plasma proteins, indicating that nitric oxide produces other reactive nitrogen species, such as nitrogen dioxide and peroxynitrite [30].

Exposure to ROS is a common occurrence for human cells, particularly for lymphocytes, which are involved in immune reactions. Excessive ROS can lead to oxidation of lipids, proteins and DNA. Both carbamylation and oxidation of proteins take place in other diseases e.g. diabetes mellitus, atherosclerosis and chronic renal failure (CRF) [19,20]. In our previous work, we showed that carbamylation and oxidation lead to decreased antioxidant potential of lymphocytes, loss of thiols and glutathione and changes in membrane fluidity [35].

The aim of this study was to evaluate the apoptotic changes and protein properties in isolated human peripheral blood mononuclear cells subjected to carbamylation, oxidation and the combination of both processes *in vitro*. Our report is based on the assessment of the activities of caspases (−2, −3, −6, −8 and −9), mitochondrial potential and the level of carbonyl, amino groups in proteins and DNA fragmentation. Moreover, the percentage of apoptotic and necrotic cells and the changes in cell morphology after treatment with cyanate and hydrogen peroxide application visualized by fluorescence microscopy were determined.

2. Material and methods

2.1. Chemicals

Sodium cyanate (NaOCN), 2,4-dinitrophenylhydrazine (DNPH), 2,4,6-trinitrobenzenesulfonic acid (TNBS), Histopaque®-1077, 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide, 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide (JC-1), propidium iodide and Hoechst 33258, 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI) were obtained from Sigma-Aldrich. RPMI 1640 bicarbonate medium, foetal bovine serum (FBS), penicillin and streptomycin were from PAA (Germany). A caspase colorimetric protease assay sampler kit (caspases-2, -3, -6, -8 and -9), catalogue number KHZ1001, was obtained from Invitrogen. All other chemicals were purchased from POCH S.A. (Gliwice, Poland) unless otherwise indicated. All dishes necessary for cell culture were obtained from NUNC.

2.2. Mononuclear blood cell isolation (MNCs)

Experiments were conducted on mononuclear blood cells isolated from the human blood buffy coat obtained from the Blood Bank in Lodz, Poland. Each single experiment was performed on cells from one donor, and n-numbers represent cells from different individuals.

Cell isolation was performed by density gradient centrifugation of blood buffy coat with Histopaque®-1077 (300 *g* for 30 min at 22 °C). The isolated cells were washed two times with phosphate buffered saline (PBS) and used for future studies.

2.3. Incubation of cells with the investigated compounds

The isolated MNCs were plated on a sterile dish (2×10^6 cells/ml). The cells were grown in RPMI medium supplemented with 10% inactivated foetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml) in a 37 °C humidified 5% CO_2 incubator.

Plated cells were incubated for 23 h with sodium cyanate (NaOCN) at a final concentration of 1 mmol/L or 2 mmol/L. At the end of the incubation, hydrogen peroxide was added to a final concentration of 100 μmol/L and incubated for 1 h longer. After incubation, cells were washed three times with PBS and used for measurement of mitochondrial membrane potential, morphological changes and DNA fragmentation. For other measurements, the cells were lysed in a buffer containing 10 mmol/L Tris, 1 mmol/L EDTA, 0.1 mol/L NaCl and 0.01% Triton X-100 (approximately 1×10^7 cells in 0.1 ml buffer).

2.4. Carbonyl content

Plasma protein carbonyl content was estimated using 2,4-dinitrophenylhydrazine (DNPH) [24]. The reaction between carbonyl groups present on proteins and DNPH leads to the formation of a stable dinitrophenyl (DNP) hydrazone product. The DNP group absorbs ultraviolet light so that the total carbonyl content of a protein or mixture of proteins can be quantified spectrophotometrically at 370 nm.

The cell lysates containing proteins were mixed with 10 mmol/L DNPH in 2.5 mol/L HCl and incubated for 1 h. At end of the incubation, 20% m/v trichloroacetic acid (TCA) was added to precipitate the protein. The precipitate was washed three times with a mixture of ethyl acetate and ethanol (1:1). Then, the protein precipitate was dissolved in 6 mol/L urea and the absorbance was measured at 370 nm. To calculate the concentration of carbonyl groups in the proteins the millimolar extinction coefficient (21.01 $mmol^{-1}$ cm^{-1}) was used. The content of carbonyls was expressed in nmol/mg protein.

2.5. Amino group content

For determination of free amino groups in lysate proteins, 2,4,6-trinitrobenzenesulfonic acid (TNBS) was used. The reaction of TNBS with amines generates a coloured product that can be readily measured at 335 nm [6].

To the samples containing 150 μg/ml of proteins, working solution (0.01% (w/v) TNBS in 0.1 mol/L sodium bicarbonate buffer, pH 8.5) was added and left for 2 h at 37 °C. Then, to stop the reaction, 10% SDS and 1 mol/L HCl was added and the absorbance measured at 335 nm.

The content of amino groups was calculated based on the calibration curve for different concentrations of homocysteine and expressed as nmol/mg protein.

2.6. Caspase activity

The activities of caspases were determined using Life Technologies™ ApoTarge™ caspase colorimetric protease assay sampler kit (caspases-2, -3, -6, -8 and -9). The caspase colorimetric protease assay sampler kit contains the substrates VDVAD (for caspase-2), DEVD (for caspase-3), VEID (for caspase-6), IETD (for caspase-8) and LEHD (for caspase-9). The substrates for caspase activity measurement are labelled at their C-termini with para-nitroaniline (pNA). The absorption of light by free pNA can be measured at 400 or 405 nm.

The comparison of the absorbance of dNA from samples allows for the determination of changes in caspase activity. The results are presented as an absorbance at 405 nm.

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