Punicalagin reduces H_2O_2-induced cytotoxicity and apoptosis in PC12 cells by modulating the levels of reactive oxygen species

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Abstract

Background: Oxidative stress has long been linked to neuronal cell death in many neurodegenerative diseases. Antioxidant conventional supplements are poorly effective in preventing neuronal damage caused by oxidative stress due to their inability to cross the blood brain barrier. Hence the use of molecules extracted from plants and fruits such as phenolics, flavonoids, and terpenoids compounds constitute a new wave of antioxidant therapies to defend against free radicals.

Objective: In this study we examined the effects of Punicalagin, a ellagitannin isolated from the pomegranate juice, on a rat adrenal pheochromocytoma cell line, treated with hydrogen peroxide, evaluating the vitality, oxidation potential, mitochondrial function and eventual apoptosis.

Methods: This study was performed on PC12 cells pretreated with Punicalagin (0.5, 1, 5, 10 e 20 µM) 24 h before of the damage by hydrogen peroxide (H_2O_2). H_2O_2 concentration (300 µM) used in our study was determined by preliminary experiments of time course. The cell vitality and ROS production were evaluated by MTS assay and cytofluorometry assays respectively. Subsequently, the number of apoptotic-positive cells and mitochondrial transmembrane potential, were measured by flow cytometry, in the same experimental paradigm. Finally, the expression of Bax and enzymatic activity of Caspase 3, some of the principle actors of programmed cell death, were investigated by semiquantitative PCR and utilizing a colorimetric assay kit respectively.

Results: We found that pretreatment with Punicalagin protected the cells from H_2O_2-induced damage. In particular, the protective effect seemed to be correlated with a

control both in radical oxygen species production and in mitochondrial functions. In fact the cells treated with H_2O_2 showed an altered mitochondrial membrane integrity while the pretreatment with Punicalagin retained both the cellular availability and the mitochondrial membrane potential similar to the control. Furthermore, the Punicalagin, modulated the apoptotic cascade triggered reducing bax gene expression and caspase 3 activity

Discussion: Results of the present study demonstrated a neuroprotective effect of Punicalagin on H_2O_2-induced PC12 cell death, including apoptosis; therefore we hypothesize a possible prevent role for this molecule in neurodegenerative diseases related to oxidative stress.

Keywords: Oxidative stress, Punicalagin, Neurodegeneration, Apoptosis, Mitochondrial dysfuction

Introduction

It has been recently assessed the pomegranate juice's ability to protect tissues and cells against stress-induced damage, with the idea that this dietary supplement may serve as a therapeutic agent [1,2]. Pomegranate possesses high amounts of ellagitannins such as punicalagin, punicalin, gallagic acid, ellagic acid and EA-glycosides [3]; in particular the punicalagin has been recently identified as a new anti-inflammatory, antiproliferative, hepatoprotective, and antigenotoxic molecule [4-6].

The concentration of hydrogen peroxide (H_2O_2) in healthy individuals is normally low, however it is frequently used as experimental model to study cellular damage by oxidative stress and to evaluate the antioxidant effects of potential new therapeutic substances. Furthermore, oxidative stress is considered as the basis of some neurodegenerative disorders such as Alzheimer and Parkinson's disease, cerebral ischemia, and other cerebral pathologies in which are produced high amounts of reactive oxygen species (ROS) [7-9]. Once ROS are generated as byproducts of cellular respiration, it is thought that mitochondria are the primary target of oxidative damage. Antioxidants can inhibit ROS formation in cells, and increase cellular defenses by up-regulating antioxidant gene transcription [10, 11].

Over the years, an accumulation of epidemiological data indicate the importance of a balanced diet rich in antioxidants to maintain proper cognitive functions and to prevent or delay neurodegeneration [12].

Within this framework, in the present study we investigated the antioxidant effects of the pre-treatment with punicalagin on an adenocarcinoma cell line (PC12), after H_2O_2-induced oxidative damage, by assessing cell viability, ROS levels and the function mitochondrial [13]. Moreover, since mitochondria play a critical role in cell apoptosis

regulation, we examined also the gene expression of Bax and activity of Caspase 3, two of the main actors of programmed cell death.

Methods

Cell Line and treatments

PC12 cells (a clonal line of rat pheochromocytoma) were cultured in a 5% CO_2 atmosphere at 37°C in RPMI with HEPES 10 mM, glucose 1.0 g/l, $NaHCO_3$ 3.7 g/l, penicillin 100 units/ml, streptomycin 100 μg/ml, 10% Fetal Calf Serum and 15% Horse Serum. Once grown until 85% confluence the cells were subcultured at an appropriate density according to each experimental procedures.

Punicalagin powder was dissolved in a 10 mM stock solutions phosphate buffer.

Direct toxicity study

For determination of vitality, PC12 cells were plated in 96-well plates at a density of 10,000 cells/well and incubated for 24 hrs, with different concentrations (50, 100, 300 and 500 μM) of H_2O_2.. Cell survival was evaluated by the 3-[(4,5-dimethylthiazol-2-yl)-5,3-carboxymethoxyphenyl]-2-(4-sulfophenyl)-2H tetrazolium, inner salt (MTS) reduction assay. The MTS assay is a sensitive measurement of the normal metabolic status of cells, which reflects early cellular redox changes. The intracellular soluble formazan produced by cellular reduction of the MTS was determined by recording the absorbance of 96-well plate using the automatic microplate photometer at a wavelength of 490 nm. Cell viability was expressed as a percentage of surviving cell. Successively, to determine the effective concentration of Punicalagin, the cells were preincubated with punicalagin at different concentration (0.5, 1, 5, 10 and 20 μM) for 24 hrs and then treated with H_2O_2 300 μM. At the light to these results, our successive investigations, were performed pretreating PC12 cells with punicalagin 10 μM for 24 h and

subsequently with hydrogen peroxide 300 μM. The morphological features of PC12 cells exposed to different treatments were analyzed and photographed by phase-contrast microscopy 40×.

Measurement of cellular generation of reactive oxygen species

The detection of ROS was performed in 96-well microplate with 25,000 cells/well. Briefly, after 24 hrs preconditioning with different concentrations (0.5, 1, 5, 10 and 20 μM) of Punigalagin, the cells were treated with H_2O_2 300 μM. After 24 hrs the cells were treated with 2',7'–dichlorofluorescein diacetate (DCFDA) which is initially non-fluorescent and is converted by oxidation to the fluorescent molecular DCF. DCF was then quantified using a CytoFluor Multi-well Plate Reader, with 485 nm excitation and 538 nm emission filters. ROS production was expressed as fluorescence intensity and expressed as a percentage of control cell.

Detection of Apoptosis

Mitochondrial membrane potential, an early marker of apoptosis induction was assessed using MitolightTM Apoptosis Detection Kit (Chemicon). Briefly, cells were cultured in the above reported experimental conditions and then incubated with the dye for 30 minutes at 37°C, as suggested by the kit protocol. The cells were placed on a microscope slide and observed immediately using a Zeiss Axiophot fluorescence microscope.

In healthy cells, the lipophilic cationic dye employed in the assay partitions to the cytoplasm and also accumulates in the form of red-fluorescent J-aggregates mitochondria, in a membrane potential-dependent fashion, due to the uptake by biochemically intact organelles. In apoptotic cells with altered mitochondrial membrane potential the dye is relocated to the cytosolic in the form of green-fluorescent monomers. Using filters to detect fluorescein and rhodamine, healthy cells are identified

as containing red mitochondria against a green background of cytoplasmic dye, while apoptotic cells appear nearly uniformly green.

In another set of experiments, the PC12 incubated with Mitolight in the conditions reported above, were immediately analyzed by Flow Cytometry (Coulter-Epics, MCL-XL). In this setting cell apoptosis was quantified by the percentage of cells detectable in the green fluorescence (FL-1) channel (a correlate for the leak of monomeric dye from depolarized mitochondria), or by their mean Green Fluorescence value.

RNA Isolation and semiquantitative PCR

Total RNA was isolated with SV Total RNA Isolation System (Promega). RNA concentration was evaluated by spectrophotometric reading at 280 and 260 nm. Total RNA was used for first strand cDNA synthesis with HyperScript, First strand Synthesis Kit and Oligo-dT, as random primer (GeneAll). The PCR was performed with about 150 ng of cDNA using DreamTaq. The following primer sequences were used for amplification:

β−Actin forward 5'-CCTTCCTGGGCATGGAGTCCTG-3',

β−Actin reverse 5'-GGAGCAATGATCTTGATCTTC-3' (208 bp);

Bax forward 5'-GCAGGGAGGATGGCTGGGGAG-3',

Bax reverse 5'-TCCAGACAAGCAGCCGCTCACG-3' (352 bp).

The experimental protocols for PCR reactions were: initial denaturation for 5 min at 95°C; amplification for 40 cycles of denaturation, 30 sec, 95°C, annealing: 30 s, at 55°C

(β−Actin), 60°C (Bax) and elongation at 72°C for 1 min; final elongation for 10 min at 72°C. PCR products were then analysed by 1.5 % agarose gels electrophoresis in TBE1X Buffer. Image acquisition and product analysis was made by Bio-Rad imaging systems with Quantity One1-D analysis software. The density of the PCR bands were divided by that of the housekeeping gene (β−Actin) and expressed as percent of the control band density.

Measurement of caspase 3 activity

Caspase-3 activity was measured by using a specific assay kit from Sigma Chemical Co. (St. Louis, MO, USA) following manufacture's instructions. DEVD-pNA was used as a colorimetric substrate. PC12 cells were plated at a density of $2,5 \times 10^6$ cells/35 mm dish and treated with 10 μM punicalagin 24 hrs before the treatment with 300 micromolar H_2O_2. After 24 h the cells were harvested by centrifugation. The pellets were washed with PBS, lysed in 50 ml of chilled cell lysis buffer and left on ice for 10 min. Lysate was centrifuged at $10,000 \times g$ for 1 min at 4°C, and supernatant was used for the caspase-3 assay.

The protein concentration was confirmed by the BCA assay. The protease activity was determined by the spectrophotometric detection at 405 nm of the chromophore p-nitroanilide (pNA) after its cleavage by caspase-3 from the labeled caspase-3-specific substrate (DEVD-pNA). The caspase 3 activity was expressed as percent of activity in the control cells

Statistical Analysis

Each experiment was repeated at least three times in single. All results were presented as the mean ± SEM of (n) replicates per experimental group. Data were subsequently

analysed by one-way ANOVA, followed by post hoc Newman–Keuls for comparisons between group means, or Dunnett test when appropriate using a PrismTM computer program (GraphPad, San Diego, CA, USA). Differences were considered statistically significant if $p < 0.05$.

Results

Effect of Punicalagin on H_2O_2-induced Cell Viability and Oxidation

First, to chose the concentration of hydrogen peroxide for using as model of oxidative injury, PC12 cells were treated for 24 hrs with different H_2O_2 concentration (50, 100, 300 and 500 μM).

As reported in Figure 1 (panel A) cell viability declined in a dose-dependent manner, reaching the 50% at 300 μM which was selected as concentration for cells related treatments.

To determine the protective effect of Punicalagin against both cell viability and H_2O_2-induced ROS production, PC12 were pretreated for 24 hrs with different concentrations of this substance (0.5, 1, 5, 10 and 20 μM) and successively insulted with hydrogen peroxide (300 μM). As evident from Figure 1 (panel B), Punicalagin pretreatment, protects the cell in a concentration-dependent manner, recovering cell viability to about 85% at 10 micromolar. No effect of punicalagin alone on cell viability was observed (data not shown). To further investigate H_2O_2 induced PC12 cell cytotoxicity and protective effect of Punicalagin, cell morphology was observed with an inverted microscope (panel C). As evident in Figure 1 (panel C), PC12 cells exhibited, respect to the control (a), a marked decrease in cell number with a lot number of cells becoming

rounded after exposure to 300 μM H_2O_2 (b). To note that the pretreatment with Punicalagin 10 μM results strongly protective (c).

Afterward, in order to investigate the effect of Punicalagin in preventing H_2O_2 induced ROS generation and the resulting oxidative stress, ROS accumulation in PC12 cells was measured. As evident in Figure 2, the level of intracellular ROS in PC12 cells treated with H_2O_2 300 μM, increases to 100% relative to the control group. The pretreatment (24 hrs) with Punicalagin at different concentration (0.5, 1, 5, 10 and 20 μM), exerts a protective effect versus ROS production, induced by H_2O_2 in a dose dependent manner. The concentration value reached of 10 μM, very similar to that of the control, it has been indicated as significantly protective.

Punicalagin pretreatment protects PC12 cells against H_2O_2-reduced mitochondrial membrane potential

In view of the tight correlation between ROS production and apoptosis (ref) we studied the effect of hydrogen peroxide exposure (300 μM) for 24 hrs in PC12 cells and the pretreatment with 10 μM punicalagin. Apoptosis detection was performed using a Green fluorescent dye which indicates the presence of depolarized mitochondria (apoptotic cells), while red fluorescence (J-aggregates) reports on functional and polarized mitochondria. As evident in Figure 3 (panel A), control cells show intense red colour due to polymerization of dye in mitochondria, indicative of healthy organelle, while in PC12 cells treated with H_2O_2 an intense green fluorescence prevails indicating a loss of mitochondrial membrane potential (Δψm) as marker of apoptosis. Pretreatment with 10 μM Punicalagin for 24 hrs significantly preserved the mitochondrial membrane potential up to a level comparable to untreated cells, indicating a protective effect able to oppose the H_2O_2-mediated apoptosis.

In order to obtain a quantitative measure of cell death and protection by Punicalagin, green fluorescence of cells labelled in the same experimental conditions as above was analysed by flow cytometry. As displayed in figure Figure 3-panel B, H_2O_2 300 μM induced apoptosis in 46 % (± 5) of PC12 cells, while pretreatment with punicalagin nearly completely prevented oxidant-induced cell death.

Expression of apoptotic gene bax

To verify the involvement of mitochondria-mediated apoptotic pathways in PC12 cells treated with 300 μM H_2O_2 we investigated the bax gene expression. As shown in figure 4 (panel A) H_2O_2 exposure for 24 hrs significantly up-regulated bax mRNA expression in PC12 cells, whereas down regulation was observed in cell pretreated with Punicalagin (10 μM for 24 hrs). No differences were observed in bcl2 gene expression (data do not shown).

Effects of Punicalagin on H_2O_2-induced Caspase-3 activation in PC12 cells.

Since caspase-3 has been shown to be an important regulator of apoptotic cell death, we next examined the effect of H_2O_2 (300 μM) on caspase-3 activity in PC12 cells. Caspase-3 has been reported to be a key performer caspase involved in cellular apoptosis which modulates the mitochondria-dependent pathway. To determine whether the protection by Punicalagin was due also to the inhibition of caspase 3, the PC12 cells were treated with H_2O_2 300 μM and Punicalagin 10 μM. As shown in Figure 4 (panel B), H_2O_2 treatment with hydrogen peroxide caused a remarkable increase of caspase-3 activity. However, adding 10 μM Punicalagin 24 hrs before H_2O_2 treatment decreased the caspase-3 activity to value similar to the control. It can therefore be concluded that Punicalagin was effective in decreasing H_2O_2-induced apoptotic cell death. This

supports the conclusion that Punicalagin inhibits H_2O_2-induced apoptosis through the regulation of intracellular ROS levels and mitochondria-dependent caspase-3 pathway.

Discussion

Oxidative stress has been implicated at the basis of the major neurodegenerative disorders [14]. Central neurons are vulnerable to insults induced by oxidative stress, due to their higher levels of polyunsaturated fatty acids and the lower levels of brain-resident antioxidants, as well as the high oxygen consumption. ROS, produced by mitochondria damaged during oxidative stress [15], can damage proteins, nucleic acids, and polyunsaturated fatty acids of cell membrane that, because of lipid peroxidation, loses its integrity with increase of permeability to Ca^{2+} [16]. Moreover, in recent years, a number of studies have shown that oxidative stress could cause cellular apoptosis via both the mitochondria-dependent and mitochondria-independent pathways [17-18]. Since these pathways are directly related to the survival or death of various cell types in normal as well as pathological situations, a clear picture of these pathways for various active molecules in their biological functions would help designing novel therapeutic strategy.

In this light, one of the plausible ways to prevent neurological disorders induced by oxidative stress is to augment or potentiate the cellular defense capacity through a proper dietary regimen intake of antioxidants.

Scientific research has shown that pomegranate juice contains higher levels of antioxidants than most other fruit juices, such as cranberry or blueberry, and even more of red wine or green tea [19]. For this reason in this study we chosen as potential protective natural molecule Punicalagin (2,3-S-hexahydroxydiphenoyl-4,6-(S-S)-gallagyl-D-glucose), an ellagitannin isolated

from pomegranate polyphenols, so has been found to protect against oxidative stress-induced cell injury in many tissues [20-22], and can increase the expression of both endogenous catalytic antioxidant systems and pro-apoptotic genes [5]. Our present study shows that the preconditioning with Punicalagin increases neuron resistance to toxicity induced by hydrogen peroxide treatment, significantly improving the cellular availability. The molecular mechanism underlying this observed phenomenon appears to be linked to a control of oxidative cellular potential, because cells pretreated with Punicalagin maintain low levels of reactive oxygen species (ROS) in contrast to cells treated only with H_2O_2. This effect is known as mediated by antioxidant enzymes; in fact numerous studies have shown that H_2O_2 activates MAP kinases in PC12 cells; MAPks mediate the production of pro-inflammatory cytokines (IL-1β, TNF-α, IL-6) and, finally, with consequent activation of the apoptosis through triggering of the caspase cascade [23]. The increase of cellular availability caused by various antioxidant agents is so related to the suppression of the MAP kinase cascade and therefore to apoptotic signals. Moreover, recent paper also showed that just punicalagin attenuates toxicity in hepatocytes by activating the nuclear erythroid 2-related factor 2 (Nrf2), a protein regulating the expression of antioxidants able to defense the cells against oxidative stress pathway, mitochondrial functions and apoptotic signals [6]. Therefore ROS are triggers of damage the mitochondrial respiratory chain and loss of mitochondrial membrane potential ($\Delta\Psi$) and all these aspects are implicated in the development of neurodegenerative diseases, which mediate or amplifying

neuronal dysfunction during the course of neurodegeneration [13,24]. In present study it was evidenced that pretreatment with Punicalagin, preserves the cells by mitochondrial membrane damage H_2O_2-induced, increasing mitochondrial membrane potential. It is known that the increased mitochondrial membrane permeability is controlled by Bax a pro-apoptotic factor, which increases the opening of the mitochondrial voltage-dependent anion channel that leads to loss of membrane potential and the release of cytochrome c [25]. Moreover, when trans-located in the mitochondrial membrane, Bax can homodimerize and triggers the activation of terminal caspases (in particular caspase 3) through alteration of mitochondrial functions, which cause the release of factors that promote apoptosis into the cytoplasm [26]. Finally, caspase-3, the main apoptotic executioner, causes chromatin condensation, protein breakdown, and DNA fragmentation [27]. On this path, we found that bax gene expression and caspase-3 activity were activated in PC12 cells by ROS production and that the pre-treatment with Punicalagin prevents all these apoptotic stimuli. Therefore, the effect of Punicalagin seems be related, in addition to a protective effect on ROS production, even at a direct inhibition of apoptotic stimuli.

In conclusion, this study shows a neuroprotective effect of Punicalagin on PC12 cell death induced by H_2O_2, also including apoptosis, resulting in a molecule with a protective potential against neurodegenerative diseases. Note, in addition, that the protective effect exerted by Punicalagin was observed with a

easily achievable concentration with a portion of juice where Punicalagin reaches levels of 2 g/L of fresh pomegranate juice [28].

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Figure Legends

Figure 1. Panel A: viability of PC12 cells following H_2O_2 different concentration (50,100,300 and 500 μM) treatment for 24 h. Panel B: viability of PC12 cells pretreated with Punicalagin at different concentration (0.5, 1, 5, 10 and 20μM), 24 h before the treatment with H_2O_2 300 μM. Cell viability was determined by MTS assay (see text). Data from six independent experiments are expressed as percent viability respect to cell untreated (control =100%) and are represented by means ± SEM.

In panel A: * and ** $p < 0.01$ and $p < 0.001$ vs controls, respectively; in Panel B: * and ** $p < 0.01$ and $p < 0.001$ vs cells treated with H_2O_2 300 μM alone.

Panel C: Morphology of PC12 cells, observed by phase-contrast microscopy . (a) Control cells; (b) cells treated with H_2O_2 300 μM for 24 h; (c) cells pretreated for 24 h with Punicalagin 10 μM and successively with H_2O_2 2 300 μM for 24 h

Figure 2. ROS production (expressed as Fluorescence Intensity) in PC12 cells pretreated with Punicalagin at different concentration (0.5, 1, 5, 10 and 20μM), 24 h before the treatment with H_2O_2 300 μM. Results are from six independent experiments and are expressed as percent viability respect to cell untreated (control =100%). All values indicate means ± SEM. * $p < 0.01$ vs cells treated with H_2O_2 300 μM alone.

Figure 3. Panel A: Mitochondrial membrane depolarization evidenced with mitolight mitochondrial kit in spectrofluorometry in PC12 cell in different experimental conditions. Red fluorescence indicates the healthy cells (polarized mitochondria), green fluorescence indicates the presence of apoptotic cells (depolarized mitochondria). Data are representative of three independent experiments. **Panel B:** Quantitative apoptotic analysis on PC12 cells. (a) Control cells; (b) cells treated with H_2O_2 300 μM for 24 h; (c) cells pretreated for 24 h with Punicalagin 10 μM and successively with H_2O_2 300 μM for 24 h. After different treatments the cells were resuspended in Mitolight solution and immediately analyzed by flow cytometer (see details in materials and methods). Data from six independent experiments are expressed as percent apoptotic cells and are represented by means ± SEM. ** $p < 0.001$ vs control cells.

Figure 4. Panel A: Apoptotic Bax gene expression in PC12 cells untreated (line 1), treated with H$_2$O$_2$ 300 mM for 24 h (line 2) and preconditioned with 10 mm Punicalagin before of treatments with H$_2$O$_2$ (line 3). Left: agarose gels representing mRNA expression levels for β-Actin (housekeeping gene) and Bax. In the first line (S) is reported DNA ladder (50 pb). Right: Bax densitometric analysis of the gels in figure above. The density of the gel bands was divided by that of the β-Actin and expressed as percent of the control band density. Data from six independent experiments are represented by means ± SEM. ** p < 0.01 vs control cells.

Panel B: Caspase-3 activity (expressed as percent of control) in PC12 cells untreated; treated with H$_2$O$_2$ 300 μM for 24 h and (c) pretreated for 24 h with Punicalagin 10 μM before the treatment with H$_2$O$_2$

Results are from six independent experiments. Significantly different from controls *P < 0.01.