Purple Sweet Potato Pigments Scavenge ROS, Reduce p53 and Modulate Bcl-2/Bax to Inhibit Irradiation-induced Apoptosis in Murine Thymocytes

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Key Words

Purple sweet potato (PSP) pigments • γ irradiation • Apoptosis • ROS • Bcl-2/Bax

Abstract

Aims: Purple sweet potato (PSP) pigments were proved to protect murine thymocytes from ^{60}Co γ -ray-induced mitochondria-mediated apoptosis in our previous study. In this study, we further investigated the effect of PSP pigments on apoptosis related ROS, p53 and Bcl-2 family. **Methods:** Cell viability was analyzed by MTT. Apoptosis was certified by DNA ladder detection. Reactive oxygen species (ROS) were detected using 2',7'-dichlorofluorescein diacetate (DCFH-DA) probe. P53, Bcl-2 and Bax proteins were analyzed by western blot. The activities of caspase-3 and caspase-9 were determined by fluorogenic substrates detection. **Results:** PSP pigments treatment prior to 4Gy ^{60}Co γ -ray irradiation increased the cell viability and decrease the apoptosis. In the presence of PSP pigments, ROS was scavenged and followed by a p53-depression. A shift in Bcl-2/Bax ratio towards anti-apoptosis was observed as a result of p53-depression. The activities of caspase-9 and caspase-3 were reduced

by PSP pigments pretreatment. **Conclusions:** PSP pigments have a cytoprotective activity against γ radiation. The protective effect of PSP pigments may be involving ROS scavenging, p53 depression and Bcl-2/Bax modulation in a caspase-dependent mitochondrial way.

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Introduction

Exposure to radiation during radiotherapy can damage normal cells as well as malignant cells. Excessive damages in normal cells bring about disturbed tissue homeostasis and a variety of pathological conditions [1]. In response to the DNA damages, cells repair the damage or enter apoptosis when the extent of DNA damages is not compatible with cell survival. Central to the DNA damage-induced apoptosis is the tumor suppressor protein p53 and its various up-stream and down-stream factors. Mice thymocytes with wide-type p53 are sensitive to

apoptotic stimuli such as γ -ray and loss of p53 function can produce resistance to γ -ray [2, 3]. ROS are reported to be an up-stream signal that triggers p53 activation and also a downstream factor that mediates apoptosis [4]. P53 targeted Bax through transcription or interacted directly with mitochondrial Bcl-2 to promote apoptosis [5, 6]. Therefore, the central role of p53 in the irradiation-induced apoptotic signal networks is of great importance.

Purple sweet potato (*Ipomoea batatas*, PSP) is regarded as an abundant source of stable anthocyanins and attracts increasing attention [7]. PSP pigments, main components as anthocyanins, are considered more beneficial in exerting their antioxidative [8, 9], anti-mutational [10] and anti-inflammatory activities [11-13], since they have no toxicity compared to synthetic compounds. Moreover, PSP pigments are found to be stable, water soluble and easily absorbed [14], which makes them suitable for further exploitation. In the present study, we investigated the effects of PSP pigments on ROS, p53 and Bcl-2 family in γ -ray-irradiated murine thymocytes.

Materials and Methods

Materials

Caspase substrates Ac-DEVD-AFC (N-acetyl-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide-7-Amino-4-trifluoromethylcoumarin) (for caspase-3) and Ac-LEHD-AFC (N-acetyl-L-leucyl-L-glutamyl-L-histidyl-L-aspartic acid amide-7-Amino-4-trifluoromethylcoumarin) (for caspase-9) were purchased from Enzyme Systems Products (Livermore, CA, USA). Bcl-2 and Bax antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). β -actin antibody was purchased from Beijing Biosynthesis Biotechnology Co. Ltd. (Beijing, China), and Horseradish peroxidase-conjugated secondary antibodies were from Wuhan Boster Biological technology Co. Ltd. (Wuhan, Hubei, China). P53 antibody, DNA Ladder, DCFH-DA, acetyl-L-cysteine (NAC), pifithrin- α , 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Bicinchoninic acid (BCA) Protein Assay Kit were obtained from Beyotime Institute of Biotechnology (Haimen, Jiangsu, China). All chemicals were of analytical grade.

PSP pigments preparation

PSP pigments, major components as cyanidin acyl glucosides and peonidin acyl glucosides (> 90%), were isolated from *Ipomoea batatas* cultivar Ayamurasaki and supplied by Qingdao Agricultural Technology Institution (Qingdao, Shandong, China). The extraction, isolation and purification by high performance liquid chromatography (HPLC) were processed in accordance with previous report [14].

A suspension of PSP pigments was dissolved in sterile deionized water and stored at 4°C.

Thymocyte preparation

Thymocyte cells were prepared from 4-week-old Kunming murine (purchased from Henan Experiment Animal Center, Zhengzhou, Henan, China). The mice were maintained under constant conditions of temperature ($23 \pm 1^\circ\text{C}$) and humidity (60%) and had free access to rodent food and tap water. The experiments were conducted in according with the guidelines of the Experimental Animal Association of China and approved by our Institutional Ethics Review Committee for Animal Experimentation. Briefly, thymus separated from Kunming mice was gently homogenised and transferred to RPMI-1640 medium containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin. Single cell suspension was made by filtered through 200 mesh nylon net under aseptic conditions. The cells were incubated in a six- or 96-well at 37°C in 5% CO₂ incubator and adjusted to 2×10^6 cells/ml. The viability of the freshly isolated cells was over 95% (as assessed by trypan blue exclusion assay).

Groups and ^{60}Co γ -ray irradiation

Thymocytes were divided randomly into several groups. Cells were incubated with indicated concentrations of PSP pigments (0.625 g/L, 1.250 g/L, 2.500 g/L), NAC (5mM) and pifithrin- α (10 μM) two hours before exposure to a single dose of irradiation with the dose rate of 0.5 Gy/min, generated from the FCC-7000 ^{60}Co γ -ray Generator (Shinva Medical Instrument Co. Ltd, Zibo, Shandong, China).

Cell viability assay

The MTT colorimetric assay of cell survival was executed on microplates. Thymocytes were seeded in 96-well microplates and treated with different doses of irradiation in order to search for the medium irradiated dose and incubated time. Different concentrations of PSP pigments (0, 0.625, 1.250, 2.500 and 5.000 g/L) were given two hours before exposure to the indicated dose of irradiation or to sham treatment. Following exposure to irradiation, cells were incubated for four hours at 37°C, and then 10 μL of MTT solution (5 mg/mL) was added to each well. Plates were then further incubated in a humidified incubator at 37°C for 4 h. By centrifugation the supernatant was rejected and 100 μL of dimethyl sulfoxide (DMSO) was added to extract the MTT formazan. Ten minutes later, the absorbance of each well was read by a multi-functional microplate reader (Tecan Group Ltd., Switzerland) at 570 nm wavelength.

DNA ladder detection

Cells were resuspended and treated in a lysis buffer (containing 150 mM NaCl, 10 mM Tris pH 7.5, 10 mM EDTA, 0.5% sodium dodecyl sulfate (SDS), 500 mg/L proteinase K) overnight at 50°C. DNA was extracted with phenol/chloroform/isopropyl alcohol (25:24:1, v/v), precipitated with sodium acetate and ethanol at -20°C overnight. Afterwards, samples were washed with 70% ethanol and treated with RNase A (20 mg/L) at 37°C for 30 min. To analysing DNA

Fig. 1. PSP pigments enhanced the cell viability of thymocytes after exposure to γ -radiation. A: Thymocytes were exposed to 0, 2, 4, 6Gy γ -irradiation respectively. Following irradiation, cells were incubated from 0 to 12 hours at 37°C. MTT assay was preformed to detect the cell viabilities. B: Thymocytes were pretreated with various doses of PSP pigments (0, 0.625g/L, 1.250g/L, 2.500g/L, 5.000g/L). Two hours later cells were exposed to 4Gy γ -irradiation or to sham-treatment. MTT assay was preformed to detect the cell viabilities. Each experiment was repeated 3 times and the data are expressed as mean $\pm$ S.D. * P < 0.05, ** P < 0.01, compared to previous concentration of PSP pigments.

laddering, samples were electrophoresed in 1.5% agarose gels containing 0.5 μ g/mL ethidium bromide. The bands were visualized with 312 nm UV radiation.

Intracellular ROS assessment

Cells were incubated for 1 h at 37°C in a CO₂ incubator after irradiation and then DCFH-DA was added at a final concentration of 0.1 mM. After incubation at 37°C for 30 min, the fluorescence was measured at emission of 530 nm with excitation of 480 nm in a multi-functional microplate reader (Tecan Safire 2, Switzerland).

Western blotting analysis

Cells were lysed in 50 mM Tris, 30 mM NaCl, 10 mM SDS, 100 mM Triton X, 6 mg/mL leupeptin, 2 mg/ml aprotinin, and 1mg/ml PMSF and boiled for 60s. Protein concentration was determined by bicinchonic acid (BCA) method. Equal amounts of total protein was fractionated by SDS-PAGE and preformed as previously described [15]. The band density was analyzed by Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA).

Caspase activity assay

Cells were resuspended in a lysis buffer (containing 1 mM EGTA, 150 mM NaCl, 50 mM Tris-HCl, 1 mM phenylmethanesulfonyl fluoride (PMSF), 10 μ g/mL aprotinin, 20 μ g/mL leupeptin, 10 μ g/mL pepstatin, 0.25% sodium deoxycholate, 0.5% Nonidet P-40) for 10 min on ice. After three freeze-thaw cycles, cellular extracts were centrifuged. Protein concentrations were determined by BCA method. The proteins were mixed with Ac-DEVD-AFC (for caspase-3) and Ac-LEHD-AFC (for caspase-9) respectively. After incubation at 37°C for two hours, the fluorescence was measured at emission of 505 nm with excitation of 400 nm in a multi-functional microplate reader (Tecan Safire 2, Switzerland).

Statistics

Data are expressed as mean $\pm$ standard deviation (SD). Statistical analysis was performed with one-way analysis of variance (ANOVA) and Q-test using SPSS software. P < 0.05 was considered statistically significant.

Results

PSP pigments enhanced the cell viability

Thymocytes were irradiated with 2, 4, 6 Gy γ -ray or sham-treated at different incubated time as shown in Figure 1A. As the irradiated doses increased, the cell viabilities were decreased correspondingly. Exposed to the same dose of irradiation, the cell viabilities declined as the incubated time extended. From 4 h post-irradiation, the cell viabilities began to decline sharply. After 4 h incubation, the cell viability was 53.1% at 4Gy γ -ray exposure.

Cell viabilities in the presence of PSP pigments at 4 h after exposure to 4Gy irradiation or sham treatment were also assessed. As shown in Figure 1B, PSP pigments prevented the decrease of cell viability induced by irradiation (P < 0.05, or P < 0.01). With 0.625-2.500 g/L PSP pigments pretreatment, the cell viability was found to increase from 61.7% to 79.7% (P < 0.05, or P < 0.01). At 5.000 g/L PSP pigments presented no statistically significant different effect on cell viability to 2.500 g/L (P > 0.05). In addition, PSP pigments did not affect cell viability under normal condition (unirradiated).

Fig. 2. PSP pigments, pifithrin- α and NAC inhibited the irradiation-induced apoptotic DNA ladder in thymocytes. Letters (a–m) indicate: Control, ^{60}Co , ^{60}Co +0.625g/L PSP, ^{60}Co +1.250g/L PSP, ^{60}Co +2.500g/L PSP, ^{60}Co +pifithrin- α , ^{60}Co +NAC groups and marker, respectively. The thymocytes were pretreated with 0.625g/L, 1.250g/L, 2.500g/L PSP pigments, 5mM NAC and 10 μM pifithrin- α two hours before 4Gy γ -ray irradiation respectively. After irradiation, the cells were incubated for six hours at 37°C and then DNA were extracted and electrophoresed on agarose gel. The marker indicated 2000, 1000, 750, 500, 250 and 100 bp.

Fig. 3. PSP pigments reduced intracellular ROS in irradiated thymocytes. Letters (a–f) indicate: Control, ^{60}Co , ^{60}Co +0.625g/L PSP, ^{60}Co +1.250g/L PSP, ^{60}Co +2.500g/L PSP and ^{60}Co +pifithrin- α groups, respectively. The thymocytes were pretreated with 0.625g/L, 1.250g/L, 2.500g/L PSP pigments and 10 μM pifithrin- α two hours before 4Gy γ -ray irradiation respectively. The cells were incubated for one hour at 37°C post-irradiation and then ROS were detected with DCFH-DA probe. The experiment was repeated 3 times and the data are expressed as mean $\pm$ S.D. # $P < 0.01$, compared to control. * $P < 0.05$, ** $P < 0.01$, compared to ^{60}Co group.

PSP pigments reduced the apoptotic DNA ladder

To study the cytoprotective effect of PSP pigments in terms of apoptosis, DNA ladder was examined. As shown in Figure 2, distinct DNA ladders appeared in ^{60}Co group. Faint DNA ladders were visible in 0.625 g/L and 1.250 g/L PSP groups. DNA smear was observed in the presence of 2.500 g/L PSP pigments. In NAC group and pifithrin- α group, DNA smear was weak and no DNA ladder was detected.

PSP pigments scavenge intracellular ROS

Over-production of ROS initiated apoptosis. The intracellular ROS level was evaluated with DCFH-

DA probe. As the shown in Figure 3, ^{60}Co irradiation doubled the intracellular ROS production ($P < 0.01$). PSP pigments pretreatment decreased ROS ($P < 0.05$ or $P < 0.01$). ROS was reduced to 65.9% in 2.500g/L PSP pigments group compared to ^{60}Co irradiated group. No difference in ROS was observed between irradiated group and pifithrin- α group.

PSP pigments down-regulated p53 protein

The p53 tumor suppressor protein is the “gatekeeper” leading to apoptosis. As shown in Figure 4A and 4B, p53 protein increased as a result of irradiation ($P < 0.05$ or $P < 0.01$). Figure 4A displayed

Fig. 4. PSP pigments down-regulated p53 protein in irradiated thymocytes. Letters (a–f) indicate: Control, ^{60}Co , $^{60}\text{Co}+0.625\text{g/L}$ PSP, $^{60}\text{Co}+1.250\text{g/L}$ PSP, $^{60}\text{Co}+2.500\text{g/L}$ PSP and $^{60}\text{Co}+\text{NAC}$ groups, respectively. A and B represent the western blot images of p53 protein. A: The thymocytes were pretreated with 0.625g/L, 1.250g/L, 2.500g/L PSP pigments two hours before 4Gy γ -ray irradiation respectively. B: The thymocytes were pretreated with 5mM NAC two hours before 4Gy γ -ray irradiation. Then the whole cell lysates were collected one hour post-irradiation and p53 protein was determined by western blot analysis. The experiment was repeated 3 times and the data are expressed as mean $\pm$ S.D. # $P < 0.05$, ## $P < 0.01$, compared to control. * $P < 0.05$, ** $P < 0.01$, compared to ^{60}Co group.

Fig. 5. PSP pigments increased Bcl-2 and decreased Bax to revert Bcl-2/Bax ratio in irradiated thymocytes. Letters (a–f) indicate: Control, ^{60}Co , $^{60}\text{Co}+0.625\text{g/L}$ PSP, $^{60}\text{Co}+1.250\text{g/L}$ PSP, $^{60}\text{Co}+2.500\text{g/L}$ PSP and $^{60}\text{Co}+\text{pifithrin-}\alpha$ groups, respectively. A and B represent the western blot images of Bcl-2 and Bax proteins. A: The thymocytes were pretreated with 0.625g/L, 1.250g/L, 2.500g/L PSP pigments two hours before 4Gy γ -ray irradiation respectively. B: The thymocytes were pretreated with 10 μM pifithrin- α two hours before 4Gy γ -ray irradiation. After irradiation, the thymocytes were incubated for four hours at 37°C and then western blot analysis was performed to detect Bcl-2 and Bax proteins. Each experiment was repeated 3 times and the data are expressed as mean $\pm$ S.D. # $P < 0.01$, compared to control. * $P < 0.05$, ** $P < 0.01$ compared to ^{60}Co group.

that p53 protein in PSP pigments was lower than in ^{60}Co group ($P < 0.01$). Figure 4B showed that p53 protein in the NAC was lower than in the ^{60}Co group ($P < 0.05$).

PSP pigments modulated Bcl-2 and Bax proteins

Bax and Bcl-2 are two of the gene products that closely related to mitochondrial apoptosis. As

shown in Figure 5A and 5B, Bcl-2 proteins were decreased by irradiation and Bax proteins were increased ($P < 0.01$). The ratios of Bcl-2 and Bax were inverted after irradiation. In the presence of PSP pigments (Fig. 5A), Bcl-2 was increased ($P < 0.05$, $P < 0.01$), except for the 2.500 g/L PSP pigments group, and Bax was decreased ($P < 0.01$). The inverted

Fig. 6. PSP pigments inactivated caspase-3 and caspase-9 in irradiated thymocytes. Letters (a–e) indicate: Control, ^{60}Co , $^{60}\text{Co}+0.625\text{ g/L PSP}$, $^{60}\text{Co}+1.250\text{ g/L PSP}$, and $^{60}\text{Co}+2.500\text{ g/L PSP}$ groups, respectively. The thymocytes were pretreated with 0.625 g/L, 1.250 g/L and 2.500 g/L PSP pigments two hours before 4 Gy γ -ray irradiation respectively. The pretreated cells were incubated for six hours at 37°C post-irradiation and the activities of caspases were tested by fluorogenic substrates detection. Each experiment was performed 3 times and the data are expressed as mean $\pm$ S.D. # $P < 0.01$, compared to control. * $P < 0.05$, ** $P < 0.01$ compared to ^{60}Co group.

ratios of Bcl-2 and Bax were reversed in the 1.250 g/L and 2.500 g/L PSP pigments groups. As shown in Figure 5B, pifithrin- α increased Bcl-2 ($P < 0.01$) and decreased Bax ($P < 0.01$) so the Bcl-2/Bax ratio was reversed.

PSP pigments inhibited the activities of caspase-3 and caspase-9

The influence of PSP pigments on caspase-3 and caspase-9 was detected to show whether the antiapoptosis effect of PSP pigments was in a caspase-dependent mitochondrial way. AFC (7-amino-4 trifluoromethylcoumarin)-derived caspase-substrates were used for the caspases activities detection. Figure 6 showed that radiation enhanced the activities of caspase-3 and caspase-9 ($P < 0.01$), and PSP pigments decreased the caspase-3 and caspase-9 activities ($P < 0.05$ or $P < 0.01$). PSP pigments reduced the activities of caspase-3 and caspase-9 to 55.8% and 61.9% respectively compared to the ^{60}Co group.

Discussion

PSP pigments, the natural anthocyanins, have attracted some attention as effective antioxidants in the recent years. Most of the PSP pigments related studies focused on the neuroprotection [11, 13, 16, 17], hepatoprotection [12, 18, 19], immunomodulation [20–22], and antiatherosclerosis [23, 24]. There has been limited information about the radioprotective effects of PSP pigments. In our previous study we have found that PSP pigments could inhibit the ^{60}Co γ -ray-induced apoptosis in murine thymocytes by mitochondrial way [15]. In the present study, we further investigated the effect of

PSP pigments on the apoptosis induced by ^{60}Co γ -ray irradiation and the roles of ROS, p53 and Bcl-2 family members involved in.

We first applied ^{60}Co γ -ray irradiation to cause DNA lesion in murine thymocytes. The cell viability assays showed that ^{60}Co γ -ray irradiation inhibited the cell survival and PSP pigments enhanced the cell viability in a dose-dependent manner without causing cytotoxicity when applied in their optimum doses. The results were in line with our previous study [15] and further demonstrated the radioprotective effects of PSP pigments. Following exposure to irradiation, a cell-specific program of apoptosis will be engaged. Here we investigated whether PSP pigments had protective effect on the ^{60}Co γ -ray-induced apoptosis. Our results indicated that the apoptosis was increased to terminate the damaged cells after irradiation. PSP pigments were found to attenuate the apoptotic DNA ladder, which suggested that PSP pigments may protect thymocytes from the ^{60}Co γ -ray-induced apoptosis. In addition, in the presence of ROS scavenger NAC and p53 specific inhibitor pifithrin- α , no DNA ladders were found, indicating that ROS and p53 played vital roles in γ -ray-induced apoptosis in thymocytes.

ROS, generated by cells as products or byproducts, are central to redox signaling and trigger apoptosis through various pathways [25]. We checked intracellular ROS after ^{60}Co γ -ray irradiation and found that ROS were doubled by irradiation and reduced significantly by PSP pigments pretreatment, which suggests that PSP pigments could scavenge ROS to rebalance the redox state. Pifithrin- α , a p53 specific inhibitor, had no influence on the hyper-level of ROS post-irradiation, which indicates that the overproduced ROS were not generated by p53 flare-up in irradiated thymocytes.

The tumor suppressor p53 plays a pivotal role in DNA damage-induced apoptosis and has close relationship with ROS [4]. P53 was investigated in our study and the results showed that the irradiation-induced p53 elevation was significantly diminished in the present of PSP pigments, which suggests that PSP pigments pretreatment may depress p53 to inhibit the irradiation-induced apoptosis. With NAC pretreatment, p53 elevation following irradiation was attenuated, suggesting that ROS stayed up-stream of p53-elevation and were one of the stimulators of p53.

P53 aims at its transcriptional target Bax [5] or translocates to mitochondria to interact with Bcl-2 in non-transcriptional way [6]. The proapoptotic Bax antagonizes the antiapoptotic Bcl-2 to reach the apoptotic balance. We evaluated Bcl-2 and Bax after irradiation and found that Bax increased sharply and Bcl-2 acted in the opposite way. Pretreatment with PSP pigments led to a significant decrease of Bax with concomitant increase of Bcl-2, resulting in a shift in Bcl-2/Bax ratio, which did not favor apoptosis. These results indicate that PSP pigments may modulate Bcl-2 and Bax towards antiapoptotic rebalance. In the present of PSP pigments, Bcl-2 and Bax alterations followed p53-depression, and pifithrin- α did not prevent Bcl-2 and Bax from antiapoptotic changes, suggesting that the p53-dependent antiapoptotic effect of PSP

pigments was through Bcl-2 and Bax modulation.

Bcl-2 and Bax control mitochondrial outer membrane permeability and thus promote the release of apoptogenic proteins such as cytochrome c, which activates caspase-9 and caspase-3 [26]. The activities of caspase-3 and caspase-9 were detected after the Bcl-2 and Bax assay. We found that caspase-3 and caspase-9 were activated by irradiation and partly blocked by PSP pigments pretreatment. These results suggest that pretreatment with PSP pigments may protect thymocytes through inactivating caspase-9 and caspase-3. These results also confirm that PSP pigments inhibit the irradiation-induced apoptosis through a caspase-dependent mitochondrial way.

Conclusion

Our study demonstrates that PSP pigments have a marked cytoprotective activity against α radiation through enhancing the cell viability and reducing the apoptosis. The protective effects of PSP pigments are in a caspase-dependent mitochondrial way and are related to ROS scavenging, p53 depression, and Bcl-2/Bax modulation.

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