Sulfated polymannuroguluronate (SPMG), a novel anti-acquired immune deficiency syndrome (AIDS) drug candidate, inhibits T cell apoptosis via combating oxidative damage of mitochondria

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Running title page
Elucidation of the protective actions of SPMG on T cells

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Abbreviations: SPMG, sulfated polymannuroguluronate; AIDS, acquired immune deficiency syndrome; HIV, human immunodeficiency virus; MMP, mitochondrial membrane potential; HPLC, high performance liquid chromatography; FITC, fluorescein-5-isothiocyanate; PMSF, phenyl methyl sulphonyl fluoride; ESI-MS, electrospray ionization-mass spectrum; ROS, reactive oxygen species; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.
Abstract

Sulfated polymannuroguluronate (SPMG) has entered the Phase II clinical trial as the first anti-acquired immune deficiency syndrome (AIDS) drug candidate in China. Herein, we report that SPMG was effective at protecting T lymphocytes against apoptosis. Further studies indicated that SPMG significantly elevated mitochondrial membrane potential (MMP) of T cells, inhibited mitochondrial release of cytochrome c (cyto c) in T cells, enhanced the activities of mitochondrial enzyme complex I, III and V, and subsequently increased ATP level and ATP/ADP ratio. In addition, SPMG potently suppressed reactive oxygen species (ROS) generation in mitochondria at cellular level and scavenged free radicals in cell free system. The molecular mechanism underlying the ATP-involved and ROS-dependent anti-apoptosis of SPMG is characterized to be due to its engagement with mitochondrial import receptor and ADP/ATP carrier (AAC) in T cell outer and inner mitochondrial membrane, respectively. All these might shed new light on the understanding of anti-AIDS functions of SPMG by protecting T cells of human immunodeficiency virus (HIV)-infected individuals.
Introduction

During human immunodeficiency virus (HIV) infection, oxidative damage and ATP level depletion will threaten T cell homeostasis and integrity and subsequently lead to apoptosis of T cells, which seems to have an adverse effect on the immune system (Pace and Leaf, 1995; Olinski et al., 2002). Apoptosis-mediated physiological depletion of T lymphocytes in the course of viral infection can silence the immune response and induce immunodeficiency (Wattre et al., 1996; Galati et al., 2002). In acquired immune deficiency syndrome (AIDS) patients, apoptotic T cell death is believed to play a major role in T cell loss and HIV pathogenesis (Gougeon et al., 1991; Ng et al., 1997). The use of antioxidant drugs in the therapy of HIV-infected patients may offer protection against oxidative stress and apoptotic cell death and slow down AIDS progression (Schreck et al., 1992; Jaruga et al., 2002). Many studies have shown that polysaccharides were capable of scavenging free radicals and protecting cells from death, which became a crucial mechanistic explanation of their anti-apoptotic actions (Liu et al., 1997; Sun et al., 2004).

Sulfated polymannuroguluronate (SPMG) is a new form of sulfated polysaccharide extracted from brown alga with an average molecular weight at 8.0 kDa. It is characterized by rich amount of 1, 4-linked β-D-mannuronate with 1.5 sulfate and 1.0 carboxyl groups averaging each sugar residue (Fig. 1A). SPMG has entered the Phase II clinical trial in China as the first marine sulfated polysaccharide with the potential of becoming an anti-AIDS drug. SPMG has manifested potential...
antiviral and immunomodulating efficacy in AIDS patients in clinical trials. Notably, our previous investigations have also verified that SPMG exerted significant immunopotentiating actions particularly on T lymphocytes (Xia et al., 2005). The goals of the present study are to investigate the anti-apoptotic and protective effects of SPMG on T cells and to elucidate the underlying mechanisms.
Materials and Methods

1. Drugs and reagents

SPMG, SPMG-Sepharose and SPMG-fluorescein-5-isothiocyanate (FITC) were provided by Marine Drug and Food Institute, Ocean University of China, China. Aprotinin, pepstatin A, leupeptin, phenyl methyl sulfonyl fluoride (PMSF), ATP, ADP, cytochrome c (cyto c), coenzyme Q1, NADH, reductive glutathione, propidium iodide (PI), rhodamine 123, dihydrorhodamine (DHR) 123, bovine serum albumin (BSA), dithiothreitol (DTT) and iodoacetamide (IAA) were purchased from Sigma (St. Louis, MO, USA). Rabbit anti-rat cyto antibody (IgG) and goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP) were purchased from Boster Biotechnology Company (Wuhan, Hubei, China). The enhanced chemiluminescence (ECL) Western blotting kit was supplied by Amersham International (Buckinghamshire, UK). RNase was obtained from Hyclone (Logan, UT, USA), RPMI 1640 medium was from Gibco (Grand Island, USA) and Trypsin (sequencing grade) from Roche (Mannheim, Germany).

2. Cell culture and preparation

Rat thymus lymphocytes were obtained from male Wistar rats of 180 to 200 g for binding and liquid chromatography (LC)-mass spectrum (MS)/MS analysis, or from male Wistar rats of about 700 g (24 months old) for anti-apoptotic and antioxidative studies following published methods (Chen et al., 1994). Cells were cultured in RPMI1640 medium supplemented with 10% fetal calf serum (FCS), 100 U/ml
penicillin and 0.1 g/l streptomycin at 37 °C in humidified 5% CO₂ incubator.

3. Apoptosis assays

3.1 PI staining

T cells prepared above were incubated with or without SPMG at final concentrations of 1, 10, and 100 mg/l for 12 h. Next the T cells were collected, washed, and stained with a mixture of RNase (1 g/l) and PI (5 mg/l) in 1 g/l sodium citrate containing 0.5% TritonX-100 (v/v) for 30 min. T lymphocytes were then harvested for apoptosis analysis using flow cytometry (FCM) (Becton and Dickinson, USA), and the percentage of hypodiploidy was analyzed with CellQuest and ModFIT LT software (Becton and Dickinson, USA).

3.2 DNA fragmentation

Thymocytes were seeded and SPMG was added at 1, 10, and 100 mg/l in culture medium. After a 12-h incubation, T lymphocytes (5 × 10⁶) were collected and washed in phosphate-buffered saline (PBS) and subsequently lysed in ice-cold lysis buffer (142.5 mM KCl, 5 mM MgCl₂, 10 mM HEPES, pH 7.3, 1 mM EGTA, 1% Triton X-100 (v/v), 0.5% Nonidet-P40 (v/v), 2.7 µM aprotinin, 0.3 µM pepstatin A, 10 µM leupeptin and 0.2 mM PMSF) for 45 min. Cellular debris were spun down and DNA was isolated from the supernatant by phenol extraction. DNA fragmentation was subsequently detected by 1% agarose gel electrophoresis to screen for the presence of a DNA ladder pattern.
4. Western blot analysis

After incubated with SPMG (1, 10, 100 mg/l) for 12 h at 37 °C, T lymphocytes were harvested and the mitochondria were removed as described below. The cytosolic extracts were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. After blocked in Tris-buffered saline (TBS) containing 0.1% Tween 20 (v/v) and 5% BSA (w/v) at room temperature for 2 h, the membranes were rinsed and incubated at 4 °C overnight with 2 mg/l anti-rat cyto c antibody (IgG) or anti-β-actin antibody as control. The membranes were then incubated with goat anti-rabbit IgG conjugated to HRP (1:2,000 dilution) at room temperature for 1 h and developed with chemiluminescence substrate, and exposed to Hyperfilm MP.

5. Measurement of intralymphocyte ATP and ADP

T cells were seeded and SPMG was added at 1, 10 and 100 mg/l respectively. After a 12-h incubation at 37 °C, T lymphocytes were harvested and the viability of cells was monitored by trypan blue exclusion method. Adenine nucleotides were extracted from samples of 1 × 10⁸ viable T cells in 0.5 M perchloric acid on ice for 10 min. After a centrifugation at 25,000 × g for 15 min at 2 °C, the supernatant was obtained and neutralized to pH 6.5–6.8. Potassium perchlorate was removed and the supernatant was stored at –80 °C. The concentrations of ATP and ADP were examined by high performance liquid chromatography (HPLC) on a CAPCELLPAK C18 SG.
column (4.6 × 150 mm, SHISEIDO, Japan). The absorbance of the eluents was monitored at 260 nm and the detector signals were recorded and integrated by a Chemstation HP software (Smolenski et al., 1998).

6. Measurement of mitochondrial membrane potential (MMP)

Alteration of the MMP of T lymphocytes after treatment with SPMG was assessed by the retention of rhodamine 123. After T lymphocytes were treated with 1, 10 and 100 mg/l SPMG for 12 h, rhodamine 123 was added at a final concentration of 1 µM and incubated for 30 min to stain the mitochondria. Next T lymphocytes were harvested and washed with PBS for three times. The changes in rhodamine 123 fluorescence were evaluated by FCM with a 488 nm laser excitation and a 530 nm emission filter.

7. Assays for mitochondrial enzyme activities

7.1 NADH dehydrogenase (complex I) assay

The activity of mitochondria complex I was measured using a modification method of Ragan et al., determining the decrease in NADH absorbance at 340 nm, which leads to the reduction of ubiquinone (CoQ1) to ubiquinol. After a 12-h incubation with 1, 10 and 100 mg/l SPMG, T lymphocytes were harvested, washed, and mitochondria were isolated as described below, followed by three cycles of freeze/thawing. The reaction was initiated by the addition of CoQ1 (50 µM) to the reaction mixture containing 20 mM potassium phosphate, pH 7.2, 10 mM MgCl₂, 0.15
mM NADH, 1 mM KCN, and the mitochondria sample. The changes of absorbance at 340 nm were examined (Cardoso et al., 1999).

7.2 Cytochrome c reductase (complex III) assay

The activity of mitochondria complex III was measured following the method of Ragan et al. This enzyme donates electrons from ubiquinol (UQ$_{1}$H$_{2}$) to cyto c, leading to the reduction of cyto c that was monitored at 550 nm. The reaction mixture contained 35 mM potassium phosphate, pH 7.2, 1 mM EDTA, 5 mM MgCl$_{2}$, 1 mM KCN, 5 µM rotenone, 15 µM cyto c and the mitochondria sample obtained as mentioned below. The reaction was initiated by addition of substrate, ubiquinol (15 µM). The changes of absorbance at 550 nm were examined (Cardoso et al., 1999).

7.3 Mitochondria ATP-synthase (complex V) assay

The enzymatic activity of ATPase in the mitochondrial inner membrane was monitored following the method of Taussky and Shorr. The mitochondria samples were incubated in 20 mM Tris-HCl (pH 7.2) containing 150 mM NaCl at 37 °C for 30 min. The reaction was initiated by the addition of 10 mM ATP and 10 mM MgCl$_{2}$, and the synaptosomes were incubated for further 20 min at 37 °C. The reaction was stopped by the addition of 5% ice-cold perchloric acid. The supernatant was obtained and the absorbance was measured at 660 nm, 15 min after the addition of molybdate reagent. The difference of the absorbance corresponds to the ATP-synthase activity (Cardoso et al., 1999; Sudo et al., 2000).
8. DHR 123 conversion assay

The level of intramitochondria reactive oxygen species (ROS) was measured with DHR 123, which can enter mitochondria of living cells and react with ROS, yielding membrane-impermeable fluorescent products. Thus, the fluorescent intensity is indicative of ROS levels within the mitochondria (Kooy et al., 1994; Dugan et al., 1995). T cells were seeded and SPMG at final concentrations of 1, 10 and 100 mg/l were added respectively. After a 12-h incubation at 37 °C, T lymphocytes were harvested, washed and loaded with DHR 123 at 10 μM in PBS containing 2 mM NaN₃ for 30 min. Then T cells were washed with PBS and cellular fluorescence was acquired using FCM with excitation at 488 nm and emission at 530 nm.

9. Chemiluminescence analysis

The scavenging ability of SPMG on superoxide radical (O₂⁻) in pyrogallol-luminol system was evaluated as follows: 100 μl PBS containing SPMG at final concentrations of 1, 10, 100, 200, 400, 600, 800, 1000 mg/l respectively or PBS alone was added into a rigid plastic tube (55 × 10 mm), followed by the addition of 50 μl pyrogallol (0.625 M). The background intensity of chemiluminescence was first tested for 10 s using a chemiluminescence analysis instrument. Then 850 μl of luminol (1 mM) was added into the tube and the intensity of chemiluminescence was measured, from which the background intensity was subtracted. The scavenging activity of SPMG on hydroxyl radical (•OH) in Vₐ-Cu²⁺-yeast suspension-luminol-H₂O₂ system
was evaluated as follows: 0.2 ml of V\textsubscript{C} (2 mM), 0.4 ml of CuSO\textsubscript{4} (2 mM), 50 µl of luminol (0.1 mM), 0.2 ml of yeast suspension (75 g/l) and 0.6 ml of SPMG solution at the same concentrations as mentioned above or PBS were added into a rigid plastic tube and mixed thoroughly. After a 30-min incubation at 37 °C, the background intensity of chemiluminescence was measured. 0.6 ml of H\textsubscript{2}O\textsubscript{2} (68 mM) was then added into the tube to start the reaction. The chemiluminescence intensity was measured and the background intensity was subtracted from it. Reductive glutathione at the same concentrations was used as control.

10. FCM and confocal microscope analysis

To detect the binding and entrance of SPMG to T cells, thymic lymphocytes were seeded and SPMG-FITC was added at 100 mg/l or not as control. After an incubation at 37 °C for 6 h, the T cells were harvested, washed three times with PBS, gated and analyzed by FCM with a 488 nm laser excitation and a 530 nm emission filter. Data were analyzed with CellQuest software. An aliquot of each sample was spotted on a slide, analyzed and photographed under a confocal laser scanning microscope (Carl Zeiss, Germany). Optical section series were collected with a spacing of 1 µm in the z-axis through T cells.

11. Determination of intramitochondria SPMG

After treated with SPMG-FITC (1, 10, 100 mg/l) for 12 h at 37 °C, T lymphocytes were harvested and washed three times with ice-cold PBS. In another
separated experiment, T cells were incubated with 100 mg/l of SPMG-FITC at 37 °C for 3, 6, 12 h, respectively. Then T lymphocytes were placed in cold isolation buffer (210 mM mannitol, 70 mM sucrose, 10 mM HEPES, pH 7.4, 1 mM EDTA, 1 mM EGTA, 10 mM KCl, 1 mM MgCl₂ and 1 mM PMSF) and hand homogenized. The homogenate was centrifuged at 1,300 × g for 5 min at 4 °C to remove nuclei and cellular debris. The supernatant was pooled and centrifuged at 10,000 × g for 10 min at 4 °C. The pellet was saved and washed three times in isolation buffer. Then pre-cooled redistilled water was added to lyse mitochondria by homogenizing on ice. The supernatant was measured by Spectrofluorometer (Jasco, Japan) with a 488 nm laser excitation and a 530 nm emission filter.

12. Purification of T cell membrane proteins

T cell membrane fraction was isolated from rat thymus lymphocytes. In brief, the thymuses were separated, minced, grinded, and filtrated. T lymphocytes were harvested, counted and centrifuged at 500 × g for 5 min in a refrigerated centrifuge. Next, they were suspended in lysing buffer [10 mM Tris-HCl (pH 7.4), 1% TritonX-100 (v/v), 150 mM NaCl, 1 mM EDTA, 1 mM PMSF and 75 units/ml of aprotinin] and left for 10 min at 4 °C. The T cells were dounce-homogenized and the lysate was centrifuged for 5 min at 1,300 × g. Then the supernatant was collected and subjected to sucrose density gradient centrifugation at 7,000 × g for 1 h. The membrane fraction located at 37–41% (w/v) sucrose was harvested and washed for three times with 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM PMSF.
(Maeda and Kashiwabara, 1996; Geetha and Deshpande, 1999).

The membrane fractions were then resuspended in solubilization buffer [20 mM Tris-HCl (pH 7.4), 2% TritonX-100 (v/v), 150 mM NaCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$, 1 mM EDTA, 1 mM PMSF] and stirred at 4 °C for 2 h, followed by centrifugation at 100,000 × g for 30 min. The supernatant was collected and applied to a SPMG-Sepharose affinity column (1 cm × 6 cm). The column was eluted with linear gradient of 0.15–2 M NaCl in Tris-HCl (pH 7.4) containing 0.1% TritonX-100 (v/v) (Maeda and Kashiwabara, 1996; Geetha and Deshpande, 1999). The protein content was estimated with 0.01% Coomassie brilliant blue G-250 (w/v) in ethanol, phosphoric acid and water (1:2:20, v/v) and the absorbance was measured at 595 nm with a microplate reader (Bradford, 1976). Fractions were pooled, dialyzed and lyophilized.

13. LC-MS/MS analysis

The proteins purified above were dissolved in 200 µl of 6 M hydrochloric carbamidine (pH 8.3), and subsequently reduced with 1 M DTT and alkylated with 1 M IAA, followed by addition of 100 mM NH$_4$HCO$_3$ and ultrafiltration at 12,000 rpm for 2 h at 4 °C. 100 µl sample was obtained and the tryptic digestion was carried out at 37 °C for 20 h. After ultrafiltration at 12,000 rpm for 90 min at 4 °C, sample was separated by a reverse-phase (C$_{18}$) capillary column (0.15 mm × 120 mm, Thermo Hypersil-Keystone, USA) and then analyzed with electrospray ionization (ESI)-MS/MS system. The mass spectrometer was set up to take one full-scan MS
from the mass range of 400–2000 m/z followed by three MS/MS spectra of the three
most intense peak. All MS/MS spectra were analyzed by SEQUEST (Thermo Finnigan,
USA) against International Protein Index (IPI) rat protein database (Bodnar et al., 2003;
Li et al., 2004).

14. Statistics

Student *t*-test and analysis of variance (ANOVA) were performed using Statview. 

*P* < 0.05 was accepted as significant and *P* < 0.01 was regarded as highly significant.

All the experiments were replicated at least for three times.
Results

SPMG protects mitochondria against cyto c release and inhibits T cells apoptosis

Numerous polysaccharides have been shown to exert anti-apoptotic activities by protecting cells from oxidative damage (Liu et al., 1997; Sun et al., 2004). More importantly, SPMG has manifested potential anti-AIDS efficacy in HIV infected individuals where apoptotic T cell death appears frequently. We therefore want to elucidate whether SPMG is able to protect T cells against apoptosis and thus explain its anti-AIDS activities in detail. Since aging T lymphocytes represent a good surrogate model for evaluation of oxidative stress- and apoptosis-involved events in mitochondria in many diseases including HIV infection (Schindowski et al., 2001; Sastre et al., 2003), thus, we selected this type of T cells in the subsequent experiments.

The percentage of hypodiploidy reflecting the degree of apoptosis was measured by flow cytometric analysis following PI staining. Results indicated that the percentage of hypodiploid cells in the control group was 40.47±5.27%. While those of hypodiploid cells in SPMG-treated groups (1, 10, 100 mg/l) were significantly lower than that of the control \( (P < 0.01) \), yielding 9.59±1.51%, 10.63±1.72% and 18.95±2.16%, respectively (Fig. 1B). Electrophoresis of endonuclease-mediated DNA fragmentation was also performed to assess the effect of SPMG on T cell apoptosis. Results showed that 1, 10 and 100 mg/l SPMG markedly suppressed DNA fragmentation and the formation of DNA ladders (Fig. 1C). All these results indicated
that SPMG at optimum concentrations exerted strong anti-apoptotic activities.

It has become clear that mitochondria play a central role in cell apoptosis by releasing mitochondrial apoptogenic proteins. Cyto c is the most important and essential component of the apoptosome (Liu et al., 1996). Therefore, we next examined the influence of SPMG on the mitochondrial release of cyto c using western blot analysis. As shown in Fig. 1D (upper panel), the cytosolic cyto c levels of the SPMG-treated groups (1, 10, 100 mg/l) were significantly lower than that of the control group, manifesting that SPMG was able to protect mitochondria and inhibit mitochondrial release of cyto c in T cells, which accounts for the anti-apoptotic activities of SPMG.

**SPMG enhances ATP level and ATP/ADP ratio in T lymphocytes**

ATP level and ATP/ADP ratio are generally accepted as a very sensitive measure for the cellular energy state (Frenzel et al., 2002). Studies indicated that the cellular ATP level is an important determination for cell death and a marker of mitochondrial activity as well. The impairment of ATP level and loss of energy charge are considered as early events in apoptosis and sufficient ATP depletion will lead to apoptotic cell death (Don et al., 2003; Comelli et al., 2003; Zhelev et al., 2004). In fact, the agents facilitating the increase in ATP and keeping it at a high level will protect cells against apoptosis (Gabryel et al., 2002). Accordingly, we want to examine if anti-apoptosis of SPMG might be attributed to the involvement of ATP. For this, we investigated the effects of SPMG on the intracellular ATP level and ATP/ADP ratio. Fig. 2A here shows
the chromatograms obtained by HPLC with isocratic elution. The incubation of T lymphocytes with SPMG (1, 10, 100 mg/l) resulted in a significant increase in the ATP contents after 12-h treatment ($P < 0.01$), manifesting a rise in the efficiency of oxidative phosphorylation. The highest elevating rate was 24.19±1.25% compared with control (Fig. 2B). The ADP level exhibited only a slight decrease ($P > 0.05$), but accompanied by a significant rise in the ATP/ADP ratio ($P < 0.01$) following SPMG treatment (Fig. 2C). These data suggested that the protective effect of SPMG is related to the preservation and restoration of high ATP levels, favoring for its ability to combat T cell apoptosis.

**SPMG promotes the MMP of T cells**

The MMP is the driving force for mitochondrial ATP synthesis and plays a decisive role in cell survival (Perl et al., 2002). The disruption of MMP can induce apoptosis by influencing oxidative phosphorylation and subsequent ATP synthesis (Nagy et al., 2003). Accordingly, we then investigated the effect of SPMG on the MMP in T lymphocytes using rhodamine 123 staining assay. FCM analysis indicated that SPMG at concentrations of 0.1, 1, 10 and 100 mg/l remarkably elevated the MMP of T lymphocytes (Fig. 3A). As shown in Fig. 3B, the fluorescent intensity of all T cells in the SPMG-treated groups (0.1, 1, 10, 100 mg/l) was 31.07±1.19 au, 39.96±1.51 au, 48.99±1.37 au and 33.90±1.42 au respectively, which were much stronger than that of the control group (17.96±0.97 au, $P < 0.01$). The cell percentage with high MMP was increased from 13.68±1.27% to 38.04±1.88%, 72.42±2.23%,
87.34±1.81% and 49.88±1.92% after SPMG (0.1, 1, 10, 100 mg/l) treatment (Fig. 3C). The enhancement of the MMP in T cells, therefore, accounts for SPMG’s anti-apoptosis action.

**SPMG enhances the activities of mitochondrial complex I, III and V**

Mitochondrial respiratory enzymes have been verified to play crucial roles during ATP synthesis. The enhancement in their enzymic activities will favor for an increase in ATP level (Du et al., 1999). SPMG was proved to elevate ATP levels in T cells and exert protective activities against apoptosis, which prompted us to hypothesize that SPMG might exhibit impact on mitochondrial respiratory enzymes. Here, we investigated the influence of SPMG on the activities of complexes I (NADH dehydrogenase), III (cytochrome c reductase) and V (mitochondrial ATP-synthase). As shown in Fig. 4A and B, SPMG significantly protected and increased the enzymatic activities of complex I and III. The absorbance reduction in the SPMG-treated groups (1, 10 mg/l) was much greater than that of the control group ($P < 0.01$). Additionally, SPMG at 1, 10 mg/l obviously enhanced the enzymatic activities of complex V. The absorbance in the SPMG-treated groups (1, 10 mg/l) (0.57±0.02, 0.58±0.02) was higher than that of the control group (0.51±0.02, $P < 0.05$) (Fig. 4C). All these data indicated that SPMG facilitated the activities of these mitochondrial respiratory enzymes, allowing the preservation and restoration of high ATP levels of T cells by SPMG.
SPMG exerts antioxidative activities

Many studies have shown that mitochondria are one of the major sources of damaging free radicals and ROS in cells and it is also a major target of these species (Cardoso et al., 1999; Sastre et al., 2003). In fact, mitochondria suffer oxidative damage more easily for its continual exposure to the accumulated ROS, which contributes to apoptosis of cells (Murphy and Smith, 2000). All these notions, together with the fact that SPMG simultaneously enhanced MMP and mitochondrial respiratory enzymes’ activities, intrigued us to presume that SPMG might exert antioxidative actions. To confirm this, we next investigated the antioxidative activities of SPMG. Flow cytometric analysis showed that SPMG treatment significantly decreased the fluorescent intensity coming from DHR conversion. The fluorescent intensity in SPMG (1, 10, 100 mg/l) groups (46.57±2.42 au, 41.83±2.34 au and 45.92±1.96 au) was much fainter than that of the control group (58.61±3.15 au, \( P < 0.01 \)), indicating the capability of SPMG to reduce aging-induced augment in mitochondrial ROS levels (Fig. 5A and B). Notably, such antioxidative activities of SPMG provided another mechanistic explanation of its anti-apoptosis activities.

Next, we further studied the free radical scavenging activity of SPMG in cell free system, with reductive glutathione as control. SPMG exerted a significant inhibition on \( \text{O}_2^- \) chemiluminescence in a concentration-dependent manner with an \( IC_{50} \) of 650 mg/l (Fig. 5C). And as shown in Fig. 5D, SPMG exhibited a strong scavenging action on \( \cdot \text{OH} \) with an \( IC_{50} \) of 450 mg/l. The inhibitory effect of SPMG on \( \cdot \text{OH} \) chemiluminescence augmented with increasing amounts of SPMG. Both of the
scavenging activities of SPMG on O$_2^\cdot$ and •OH were a little lower than those of reductive glutathione. It was also shown that SPMG had a stronger scavenging activity on •OH than that on O$_2^\cdot$. The free radical scavenging abilities of SPMG might block ROS generation from the initial and thus reduce the accumulation of ROS in mitochondria, accounting for the antioxidative activities of SPMG.

**SPMG enters T cell mitochondria**

It is known that polysaccharides can exert their bioactivities via directly binding to receptors or partners in immunocytes (Honda et al., 1994; Willment et al., 2001). All these led us to postulate that there may be SPMG binding sites (receptors) in T lymphocytes. We first examined the possible binding of SPMG to T cells. FCM analysis showed that the fluorescent intensity of T lymphocytes in the SPMG-FITC group (22.46±1.41 au) was much stronger than that of the control group (7.72±0.23 au, P < 0.01) (Fig. 6A). We then confirmed this finding using confocal microscopy analysis. As shown in Fig. 6B, T lymphocytes of the SPMG-FITC group showed bright greenish-yellow fluorescence, while control group exhibited no fluorescence, indicating a significant amount of SPMG binding in T lymphocytes. Notably, the confocal slicing up analysis further verified that SPMG entered into T cells and was engaged with binding partners in cell cytoplasm (Optical sections of 5 µm in the z-axis through the center of T cells were shown). All these observations then raised the possibility that SPMG might enter into the mitochondria.

We then confirmed the possibility of SPMG entering into the mitochondria with
FITC labeled probe of SPMG. Data from spectrofluorometer examination showed that after incubation with SPMG-FITC, the fluorescent intensity of mitochondria homogenate was enhanced gradually compared with control (Fig. 6, C and D). The increase in incubation time (from 3 to 12 h) and SPMG concentration (from 1 to 100 mg/l) resulted in incremental increase in the fluorescent intensity of the mitochondria homogenate of T cells (from 0.39±0.07 to 5.47±0.22, and from 1.39±0.09 to 5.47±0.22, respectively). These data confirmed the entrance of SPMG into T cell mitochondria in a time- and concentration-dependent manner. The capability of SPMG to target mitochondria, together with its antioxidative actions, can effectively protect mitochondria from oxidative damage and subsequently protect T cells against apoptosis.

**SPMG binds to the mitochondrial import receptor and ADP/ATP carrier (AAC) in T cell mitochondrial membrane**

The above studies substantially supported that SPMG entered into the mitochondria. We next want to identify and characterize the possible engagement of SPMG with mitochondria. For this, membrane protein preparation was first obtained from T lymphocytes. After solubilization, the preparation was applied to the SPMG-Sepharose affinity column and eluted with linear gradient of NaCl (0.15–2 M) in Tris buffer. As a result, numerous proteins were eluted at NaCl concentrations between 0.15 and 1 M. At the range from 1 to 2 M of NaCl, a single symmetric peak with relatively less proteins was obtained, which was subsequently applied to
LC-MS/MS analysis (Fig. 7A).

A full-scan spectrum from LC-MS/MS analysis is shown in Fig. 7B. It was confirmed by MS/MS analysis that SPMG can engage both with the mitochondrial import receptor and AAC, which lie in the outer and inner mitochondrial membrane, respectively. Table 1 lists all the identified peptides by MS/MS matching these two proteins. The MS/MS spectrum of the mass peak at m/z 2372.61 of mitochondrial import receptor, and that at m/z 2798.10 which is involved in AAC are shown in Fig. 7, C and D respectively as examples. The binding of SPMG to these two transporting receptors in both outer and inner membrane of mitochondria might give us a good explanation of the transporting mechanism of SPMG into the mitochondria.
Discussion

The key function of mitochondria in cells is to provide ATP by oxidative phosphorylation (Saraste, 1999; Perl et al., 2002). Mitochondria are also a major source of free radicals and ROS and a target for their damaging effects (Frenzel et al., 2002; Fang and Beattie, 2003). A large body of evidence indicates that during aging, free radical and ROS productions by mitochondria sharply increase. These findings are accompanied by the impairment in mitochondrial function and morphology, including a decline in MMP, an increase in mitochondrial release of apoptogenic molecules, a decrease in activities of electron transport chain complexes, and a subsequent depletion of mitochondrial energy production (Schindowski et al., 2000; Sastre et al., 2003). All these as a consequence trigger cell apoptosis to a great extent both in vivo and in vitro (Schindowski et al., 2001). Therefore, cells from aging rats have been accepted as a good model for evaluating the mitochondrial oxidative stress-associated apoptosis.

T lymphocytes infected with HIV can enhance production of ROS, which may result in severe oxidative stress in HIV-infected patients. Such increase in oxidative damage can cause the depletion of ATP level and loss of energy charge, threatening T cell homeostasis and integrity and leading to apoptotic cell death of patients. All these favor further viral replication and accelerate the progression of AIDS (Pace and Leaf, 1995; Olinski et al., 2002). Strategies to prevent mitochondria from oxidative damage and to restore mitochondrial functions may provide new therapies for HIV infection.
In fact, a very wide range of antioxidants have been claimed to inhibit HIV infection in T cells and offer protection against the development of AIDS (Schreck et al., 1992; Jaruga et al., 2002). It is conceivable that compounds increasing ATP supply will exert anti-apoptotic functions against severe T cell depletion caused by HIV infection (Gabryel et al., 2002). In our studies, the SPMG supplementation significantly enhanced ATP/ADP ratio and kept ATP at a high level. This finding supports a theory that the maintenance of ATP energy supply accounts for the anti-apoptotic activities of SPMG.

The MMP is increasingly recognized to be the driving force for mitochondrial ATP synthesis and play a decisive role in cell survival (Perl et al., 2002). Disruption of MMP is thought to be a significant factor in the induction of apoptosis (Nagy et al., 2003). SPMG dramatically increased the MMP of T cells, accompanied by the reduction of the cyto c release from mitochondria. We suspect that this mechanism underlies the abilities of SPMG to promote ATP synthesis and protect T cells against apoptosis. In addition, mitochondrial respiratory enzymes have also been verified to play crucial roles in ATP synthesis. The enhanced enzymic activities will facilitate the efficiency of oxidative phosphorylation and consequent ATP level and energy charge (Du et al., 1999; Monteiro et al., 2004). In the present study, SPMG potently increased the activities of respiratory enzymes including complex I, III and V, which may give us a good explanation of the elevated actions of SPMG on ATP levels in T cells.

The mitochondrial electron transport chain is an important source of ROS, which in turn drives the mitochondria to be continually exposed to the accumulated ROS.

(Murphy and Smith, 2000).
Therefore, the mitochondria are more vulnerable to suffer from the oxidative damage than the rest of the cells (Cardoso et al., 1999; Murphy and Smith, 2000; Fang and Beattie, 2003). In fact, ROS-induced collapse of MMP, mitochondrial release of apoptotic factors including cyto c, and intracellular ATP depletion are commonly accepted as the causes of apoptotic cell death (Frenzel et al., 2002, Comelli et al., 2003, Nagy et al., 2003). Numerous polysaccharides have been identified to scavenge free radicals and protect cells from death, due to their ability to degrade the excessive free radicals and ROS (Liu et al., 1997). SPMG protected mitochondria from oxidative damage by targeting mitochondria and scavenging free radicals including O$_2^{-}$ and •OH effectively. These abilities of SPMG might block ROS generation from the initial and thus reverse the accumulation of ROS in mitochondria, accounting for the antioxidative and anti-apoptotic activities of SPMG.

Increasing evidence have highlighted that polysaccharides can exert their bioactivities via directly binding to receptors or partners in immunocytes (Honda et al., 1994; Willment et al., 2001). In the present studies, we found that SPMG not only binds to and enters into T cells, but also targets the mitochondria. Further investigation using SPMG affinity chromatography and subsequent LC-MS/MS analysis supported a theory that SPMG binds to both mitochondrial import receptor and ADP/ATP carrier protein, which lie in the outer and inner membrane of mitochondria respectively. These notions indicate that SPMG’s targeting on T cell mitochondria is a receptor-mediated event, which in turn underlies the anti-apoptotic activities of SPMG on T cells.

In summary, we have demonstrated for the first time that SPMG exhibited
anti-apoptosis of T cells via targeting mitochondria by scavenging free radicals and decreasing ROS accumulation, and thus protecting mitochondria against cyto c release and improving the ATP energetic status. The restoration of SPMG on ATP depletion is likely due to its enhancement in MMP and activation of the mitochondrial respiratory enzymes via binding to the mitochondrial importer receptor and AAC receptor in mitochondria. All these effects might shed new light on understanding the anti-AIDS activities of SPMG, particularly its correction of the immune deficiency and reversal of the excessive T cell depletion upon HIV infection. The explicit mechanisms of SPMG underlying apoptosis-involved physiological depletion of T lymphocytes in the course of viral infection need to be further elucidated, which will provide proof of principle for SPMG in immune silencing-associated anti-AIDS function.
References


lymphocytes of HIV infected patients. CR Acad Sci III 312:529–537.


Footnotes:

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Figure legends:

**Fig. 1.** Effects of SPMG on apoptosis of T cells and mitochondrial release of cyto c. Thymocytes were incubated with SPMG (1, 10, 100 mg/l) for 12 h at 37 °C and then stained with PI (5 mg/l) and analyzed by FCM, The proportion of T cells in the sub G₀ fraction representing apoptotic cells was shown (B). For DNA fragmentation analysis, SPMG (1, 10, 100 mg/l) treated T lymphocytes were lysed in ice-cold lysis buffer and cellular debris were spun down. DNA was isolated and detected by 1% agarose gel electrophoresis to screen for the presence of a DNA ladder pattern (C). To determine the influence of SPMG on mitochondrial release of cyto c, cytosolic fractions were extracted from SPMG (1, 10, 100 mg/l) treated T cells and subjected to Western blotting analysis using anti-cyto c antibody. As a control for equal loading of proteins, filter was also probed with anti-β-actin antibody (D). Four parallel samples were prepared in each group and the result shown is a representative of three separate experiments with similar results. A, structure of SPMG; B1, control; B2, 1 mg/l SPMG; B3, 10 mg/l SPMG; B4, 100 mg/l SPMG.

**Fig. 2.** Effects of SPMG on the ATP level and ATP/ADP ratio in T lymphocytes. SPMG (1, 10, 100 mg/l) treated T lymphocytes were harvested and adenine nucleotides were extracted in 0.5 M perchloric acid on ice for 10 min. After neutralized to pH 6.5~6.8, the supernatant was examined by HPLC on a CAPCELLPAK C18 SG column. The absorbance of the eluents was monitored at 260
nm (A). Four parallel samples were prepared in each group and the data shown is a representative of three independent experiments with similar results. A1, standard sample; A2 control; A3, 1 mg/l SPMG; A4, 10 mg/l SPMG; A5, 100 mg/l SPMG; B, comparison of ATP content; C, comparison of ATP/ADP ratio. **, \( P < 0.01 \) comparing with control.

**Fig. 3.** Effect of SPMG on the MMP of T cells. T lymphocytes were incubated with 1, 10 and 100 mg/l SPMG for 12 h, rhodamine 123 was added at a final concentration of 1 µM and incubated for 30 min. Next T lymphocytes were harvested, washed and evaluated by FCM (A). The fluorescent intensity of all T cells (B) and the percentage of the T cells with high MMP (C) were shown respectively. Four parallel samples were prepared in each group and the data shown is a representative of three separate experiments with similar results. A1, control; A2, 0.1 mg/l SPMG; A3, 1 mg/l SPMG; A4, 10 mg/l SPMG; A5, 100 mg/l SPMG **, \( P < 0.01 \) comparing with control.

**Fig. 4.** Effects of SPMG on Complex I, III, and V activities. T cell mitochondria were isolated after treated with SPMG (1, 10, 100 mg/l) as mentioned in methods. Mitochondria complex I activity was measured by the addition of CoQ1 (50 µM) to the reaction mixture and the changes of absorbance at 340 nm were examined (A). After the reaction was initiated by addition of 15 µM ubiquinol to the reaction mixture, the activity of mitochondria complex III was measured by examining the changes of
absorbance at 550 nm (B). To examine the activity of complex V, the reaction was initiated by the addition of 10 mM ATP and 10 mM MgCl₂, and stopped by the addition of ice-cold 5% perchloric acid. The supernatant was obtained and the absorbance was measured at 660 nm after the addition of molybdate reagent (C). Four parallel samples were prepared in each group and the data shown is a representative of three independent experiments with similar results. *, P < 0.05 and **, P < 0.01 comparing with control.

**Fig. 5.** Analysis of antioxidative activities of SPMG. SPMG at 1, 10 and 100 mg/l were added to T cells and incubated for 12 h at 37 °C, then T lymphocytes were harvested and loaded with 10 µM of DHR 123 for 30 min at 37 °C. Next T cells were washed with PBS and cellular fluorescence was acquired using FCM (A and B). The scavenging ability of SPMG on superoxide radical (O₂⁻) was evaluated in pyrogallol-luminol system (C) and the scavenging activity of SPMG on hydroxyl radical (•OH) was evaluated in Vc-Cu²⁺-Yeast suspension-luminol-H₂O₂ system (D). Reductive glutathione at the same concentrations was used as control. Four parallel samples were prepared in each group and the data shown is a representative of three independent experiments with similar results. A1, blank; A2, control; A3, 1 mg/l SPMG; A4, 10 mg/l SPMG; A5, 100 mg/l SPMG. ##, P < 0.01 comparing with blank and **, P < 0.01 comparing with control in B.

**Fig. 6.** Binding and entrance of SPMG to T cells and to T cell mitochondria.
Thymocytes were seeded and SPMG-FITC was added at 100 mg/l. After 6 h incubation at 37 °C, T cells were harvested, washed, and analyzed by FCM (A). An aliquot of each sample was spotted on a slide, analyzed and photographed under a confocal laser scanning microscope. Optical sections of 5 µm in the z-axis through the center of T cells were shown (B). To elucidate the entrance of SPMG into T cell mitochondria, T lymphocytes were incubated with 1, 10, 100 mg/l of SPMG-FITC for 12 h at 37 °C (C) or with 100 mg/l SPMG-FITC at 37 °C for 3, 6, 12 h, respectively (D). Next T cells were harvested, washed, homogenized, and mitochondria were isolated and lysed by homogenizing on ice. The supernatant was obtained and measured by Spectrofluorometer. Four parallel samples were prepared in each group and the result shown is a representative of three separate experiments with similar results. 1, control; 2, SPMG-FITC. **, $P < 0.01$ comparing with the front group in C and D.

**Fig. 7.** Purification and LC-MS/MS analysis of SPMG binding mitochondrial import receptor and ADP/ATP carrier protein. The solubilized thymocyte membrane preparations were applied to a SPMG-Sepharose affinity column and the SPMG-bound proteins were eluted with 0.15–2 M linear gradient of NaCl in Tris buffer (pH 7.4) containing 0.1% TritonX-100 (v/v). The eluted proteins were estimated with 0.01% (w/v) Coomassie brilliant blue G-250 in ethanol, phosphoric acid and water (1:2:20, v/v) and the absorbance was measured at 595 nm (A). The fraction 2 with higher
affinity was applied to LC-MS/MS analysis. The proteins were reduced, alkylated and digested. After separated by a reverse-phase capillary column, the sample was analyzed by ESI-MS/MS. The data shown is a representative of three independent experiments with similar results. A, the elution curve of the membrane proteins binding to SPMG; B, the full-scan spectrum by LC-MS/MS analysis; C, MS/MS spectrum of the mass peak at m/z 2372.61 of mitochondrial import receptor; D, MS/MS spectrum of the mass peak at m/z 2798.10 of AAC.
TABLE 1

LC-MS/MS characterization of SPMG binding mitochondrial import receptor (A) and ADP/ATP carrier protein (B).

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<th>Peptide sequence</th>
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<th>Delta Cn</th>
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</table>
A

![Chemical structure diagram]

\[ R = \text{SO}_3^- \]

B

1. M1: 40.47 ± 5.27%
2. M1: 9.59 ± 1.51%
3. M1: 10.63 ± 1.72%
4. M1: 18.95 ± 2.16%

C

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D

<table>
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<tr>
<td>10</td>
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IB: anti-Cyto c
IB: anti-β-actin