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Proteomic Analysis of Pycnogenol Effects in RAW 264.7 Macrophage Reveals Induction of Cathepsin D Expression and Enhancement of Phagocytosis

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Pycnogenol, polyphenolic compounds extracted from the pine bark, is beneficial for human health. To understand more of its effects, the present study is to explore the protein expression pattern induced by pycnogenol in RAW 264.7 cells. Global analysis using two-dimensional gel electrophoresis indicated that treatment with pycnogenol induces upregulation of four proteins, whose identities were revealed by mass spectrometry as cathepsin D, keratinocyte lipid-binding protein, proteasome subunit alpha type 1, and annexin IV. The pycnogenol effect displayed a time- and concentration-dependent manner. Unlike pycnogenol, N-acetyl cysteine and vitamin C had no effect on cathepsin D expression. Further studies showed that cathepsin D induction is correlated with an increase of lysosomal staining and enhancement of phagocytosis. These results reveal the novel effects of pycnogenol on protein expression and phagocytic functions and illustrate the advantage of proteomics-based strategy in unveiling the molecular basis of phytochemicals.

KEYWORDS: Pycnogenol; proteomics; macrophage; cathepsin D; phagocytosis

INTRODUCTION

Several lines of evidence obtained from epidemiological and biomedical studies have indicated that phytochemicals extracted from fruits, vegetables, and plants are beneficial for human health (*1*, *2*). Pycnogenol is a nutritional supplement extracted from the bark of *Pinus maritima* and mainly composed of procyanidins and phenolic acids (*3*, *4*). Because of the consistency of its phytochemical composition by standardized extraction processes, pycnogenol represents a suitable and convenient model to study the effect and mechanism of phytochemicals. Previous studies in humans indicated that pycnogenol has favorable effects on the cardiovascular system, diabetes, pain relief, skin protection, immunomodulation, and asthma (*3–5*). As with other phytochemicals, it is thought that pycnogenol effects are primarily mediated by its ability to scavenge free radicals and to inhibit free radical-producing enzymes (*3–5*).

In addition to antioxidant activity, recent studies were concentrated on the regulatory effects of pycnogenol on inflammation-mediated events. Pycnogenol has been shown to increase tumor necrosis factor-α (TNF-α) secretion and to block the expression of interleukin-1 and inducible nitric oxide synthase in RAW 264.7 macrophage (*6–8*) and interleukin-2 in Jurkat T cells (*9*). Inhibition of expression of adhesion molecules in vascular endothelial cells and keratinocytes was documented as well (*10*, *11*). Together these observations demonstrate the immunomodulatory effects of pycnogenol.

Thus, pycnogenol produces a wide spectrum of effects and is able to exert its effect through diverse pathways. We are interested in phytochemical research and have successfully applied proteomics technology to study the anticancer effect of *Antrodia camphorata* in lung cancer cells (*12*). To better understand the immunomodulatory effects of pycnogenol, we employed two-dimensional (2D) gel electrophoresis in combination with mass spectrometry to examine the proteome of mouse RAW 264.7 macrophage and search for proteins whose expression is regulated by pycnogenol. RAW 264.7 cells were chosen as a model in the present study because effects of pycnogenol on antioxidation and gene expression have been documented in this cell line (*6–9*). Using proteomic approaches, four proteins were found to be upregulated by pycnogenol. Protein identification by mass spectrometry revealed the identities of these spots and the effects of pycnogenol on cellular functions were further explored.

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MATERIALS AND METHODS

Materials. The primary antibodies utilized in this study were obtained from the following sources: cathepsin D (Upstate Biotech, Charlottesville, VA; R&D systems, Minneapolis, MN; annexin IV (Lab Vision, Fremont, CA); proteasome subunit alpha type 1 (Stressgen Bioreagents, Victoria, British Columbia, Canada); actin (Chemicon international, Temecula, CA). Horseradish peroxidase-conjugated secondary antibodies were from Amersham Pharmacia Biotech (Piscataway, NJ). Fetal bovine serum and DMEM medium were purchased from Invitrogen (Carlsbad, CA). IPG gel strips and reagents for isoelectric focusing were from Amersham Pharmacia Biotech (Piscataway, NJ), and those for SDS–PAGE and Western blotting were from Amresco (Solon, OH). Chemiluminescence reagents, 2D clean kit, 2D plus silver stain kit, and gel strips were from Amersham Pharmacia Biotech (Piscataway, NJ). Trypsin was from Promega (Madison, WI). Pycnogenol was a generous gift from Horphag Research (Geneva, Switzerland). LysoSensor Green DND-189 was from Invitrogen (Carlsbad, CA). All other agents were purchased from Sigma (St. Louis, MO).

Cell Culture and Pycnogenol Treatment. RAW 264.7 mouse macrophage cell line was purchased from American type Culture Collection and cultured at 37 °C in DMEM supplemented with 10% fetal bovine serum. A stock solution (50 mg/mL) of pycnogenol was prepared in water, and a final concentration of 100 μg/mL was utilized throughout the experiments unless otherwise noted. Incubation times were 24 h or as indicated in time-course studies.

Sample Preparation for 2D Gel Electrophoresis. In this regard, cells in T75 flask treated for 24 h with or without 100 μg/mL pycnogenol were harvested, washed, and lysed in lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1 mM EDTA, 1 mM PMSF, 100 U/mL aprotinin, 100 nM dithiothreitol). Samples were then centrifuged at 14000*g* for 20 min. The supernatant was collected and centrifuged again at 75 000 rpm in Optima ultracentrifuge (Beckman). After centrifugation, the supernatant was cleaned with a 2D clean kit, and the protein pellet was dissolved in rehydration buffer and stored at −80 °C.

2D Gel Electrophoresis. The immobilized pH gradient (IPG) strips (pH 4–7, 11 cm) were rehydrated for 16 h with 200 μL of rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 2% dithiothreitol, 0.5% IPG buffer, and a trace amount of bromophenol blue) containing 60 μg of protein. The proteins were then focused at 200, 1000, and 8000 V with a total of 32 000 voltage-hours. After isoelectric focusing, the gel strips were equilibrated in equilibration buffer (6 M urea, 30% glycerol, 2% SDS) containing 2% dithiothreitol for 15 min and then in equilibration buffer containing 5% iodoacetamide for a further 15 min. The equilibrated gels were loaded onto the top of acrylamide gel (12.5%) and sealed with 0.5% agarose. The proteins were separated until bromophenol blue reached the bottom of the gel.

Silver Staining. The 2D plus one silver stain kit was utilized to detect proteins, using a modified protocol as described elsewhere (*12*). Briefly, the gel was fixed in fixation solution (ethanol/water/acetic acid, 4/5/1, v/v) after electrophoresis and treated with sensitizing solutions (0.5 M sodium acetate, 0.5% sodium thiosulphate) for 30 min. After sensitization, the gels were washed and incubated in 0.25% silver nitrate solutions for 20 min and then developed by incubating with the developing solution (2.5% sodium carbonate and 0.015% formaldehyde).

Image Analysis. To look for proteins showing disparity in expression, the proteome maps were analyzed by PDQuest (BioRad) software. A total of eight pairs of well-focused gels from control and pycnogenol-treated samples were compared. The intensity of differentially expressed spots was calculated and normalized as % of total volume, corresponding to the intensity of each spot divided by the sum of intensities of all spots in the gel, and furthered analyzed by a *t*-test. In all cases, a P value less than 0.05 was considered as significantly different.

In Gel Digestion. Protein spots excised from the 2D gels were digested by trypsin as described previously (*12*). Briefly, the spots were destained and treated with 50 ng of modified trypsin in 100 μL of 25 mM ammonium bicarbonate at 37 °C overnight. The supernatant was collected after digestion, and the gel was extracted with 200 μL of 0.1% formic acid. Then the extracts were combined, dried, and resuspended in 0.1% formic acid immediately for mass spectrometric analysis or stored at −20 °C until use.

Liquid Chromatography-Tandem Mass Spectrometry. Protein identification was carried out by LCQ Deca ion trap mass spectrometer (ThermoFinnigan, San Jose, CA) interfaced with an Agilent 1100 liquid chromatography system. The digested peptides were separated in Agilent ZORBAX 300SB-C18 column (150 × 0.3 mm, 3 μm particle diameter, 300 Å pore size) using a mobile phase of solution A (0.1% formic acid in water) and solution B (0.085% formic acid in acetonitrile). The peptides were eluted at a flow rate of 5 μL/min with an acetonitrile gradient consisting of 5–16% solution B in 5 min, 16–20% solution B in 40 min, and 20–65% solution B in 40 min. The eluted peptides were introduced online to the ionization source, and the spectra were acquired as successive sets of three scan modes (MS, Zoom, and MS/MS scans) as described previously (*12*). The acquired collision induced dissociation spectra were interpreted with TurboSequest software (ThermoFinnigan, San Jose, CA) that matches predicted tandem mass spectra from the database. Generally, a cross correlation value greater than 2.0, a correlation score higher than 0.1, and Rsp < 5 were considered as a significant match.

Immunoblotting. After incubation with or without pycnogenol for 24 h or for various times as indicated, RAW 264.7 cells were harvested and lysed in SDS–PAGE sample buffer. Total cell lysates (50 μg of protein) were separated by gel electrophoresis and transferred onto membrane. The membrane was blotted with primary antibodies overnight and then with secondary antibodies (1:15 000), followed by visualization with chemiluminescence.

Lysosomal Staining. RAW 264.7 cells incubated in the presence or absence of 100 μg/mL pycnogenol for 24 h were washed with DMEM and stained for 1 h with LysoSensor Green DND-189 dye. The dye emits fluorescence in acidic environment such as lysosome and phagosome. After staining, cells were washed three times with phosphate-buffered saline and analyzed by confocal microscopy (Leica TCS SP2). The wavelengths for excitation and emission are 443 and 505 nm, respectively.

Phagocytosis Assay. In the present study, phagocytosis is determined by the amount of fluorescence-labeled latex beads internalized by RAW 264.7 cells. For microscopic analysis, cells were treated with or without 100 μg/mL pycnogenol overnight, incubated with fluorescent latex beads (Sigma, L-3030, carboxylate-modified, average size 2 μm, 10 μL beads in 2 mL of DMEM) for 2 h, and then analyzed by confocal microscopy as described above with excitation at 543 nm and emission at 610 nm. For flow cytometry analysis, cells were treated as described above and detached by pipeting after washing in phosphate-buffered saline, and the fluorescence was measured by FACSCalibur flow cytometer (Becton-Dickenson, San Jose, CA). The cytographs shown in **Figure 6B** represent an analysis of 10 000 cells per sample.

MTT Assay. Various concentrations of pycnogenol as indicated in **Figure 8** were added to RAW 264.7 cells in a 96-well plate. After treatment for 24 h, 20 μL of MTT solution (5 mg/mL, Merck, Darmstadt, German) was added to each well and incubated for a further of 4 h. The medium was then aspirated and 200 μL per well of DMSO was added, and the plate was gently shaken for 5 min before the absorbance was determined at 540 nm. Triplicate wells were conducted for each concentration of pycnogenol tested in this study. Values obtained from cells not treated with pycnogenol were employed as the control, and pycnogenol's effects were normalized and illustrated as % of control.

RESULTS

Molecular Profiling of Pycnogenol-Induced Protein Expression in RAW 264.7 Macrophage. As described above, results from in vivo and in vitro studies suggest a role of pycnogenol in modulation of immune response. To further explore pycnogenol effects in a cellular model, we employed 2D gel electrophoresis to separate proteins and investigated differential protein expression after 24 h treatment of pycnogenol (100 μg/mL) in mouse macrophage RAW 264.7 cells. At 100

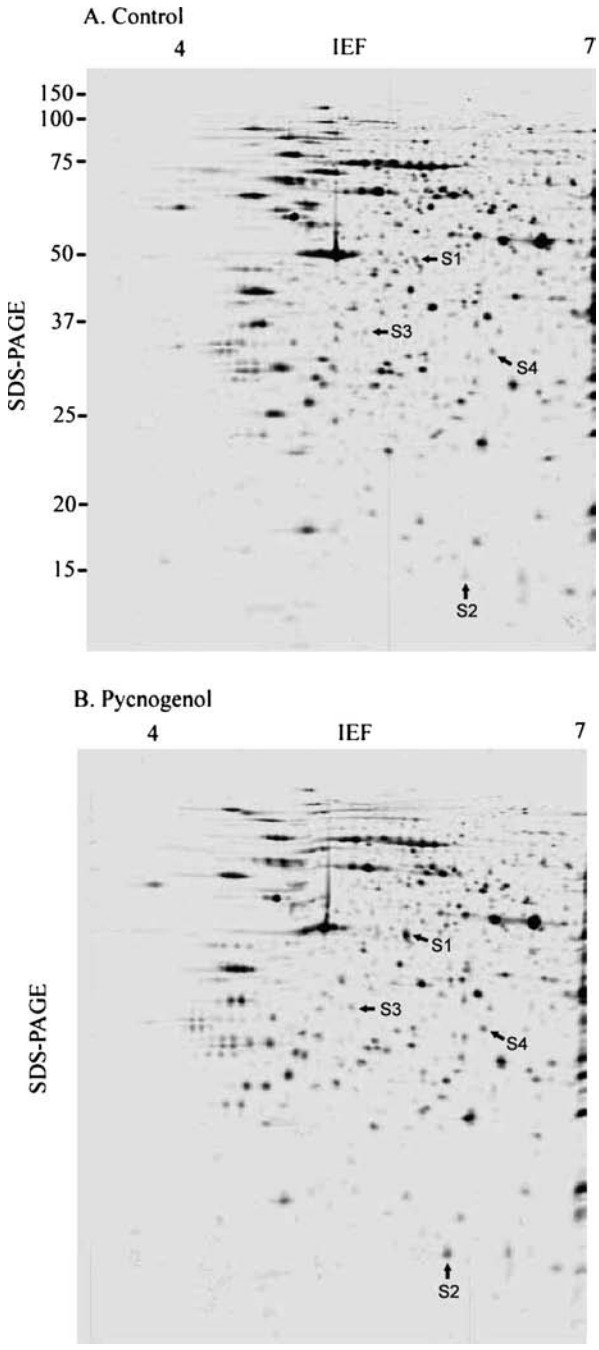

Figure 1. (**A**, **B**) Profiling of protein expression in RAW 264.7 cells by 2D gel electrophoresis. Cells treated with or without 100 μg/mL pycnogenol for 24 h were harvested and processed for 2D gel electrophoresis as described in Materials and Methods. A total of 60 μg of proteins were loaded onto the IPG gels (11 cm, pH 4–7) for isoelectric focusing (IEF) and then to the 12.5% acrylamide gel for SDS–PAGE, after which proteins were visualized by silver staining. Numbers shown on the top indicate the pH gradient, whereas those on the left indicate the molecular mass (kDa) of protein standards. There are about 700 spots detected by imaging analysis. The spots illustrating differential expression are indicated by arrows and denoted by numbers S1–S4. Gels shown are from experiments conducted simultaneously and representative of eight independent experiments.

μg/mL, pycnogenol produced potent or near-maximal effect on TNF-α secretion, regulation of gene expression, free radical scavenging, and inhibition of free radical-producing enzymes (*6, 8, 10, 11*). **Figure 1** illustrated two representative proteome maps of RAW 264.7 cells treated without or with pycnogenol

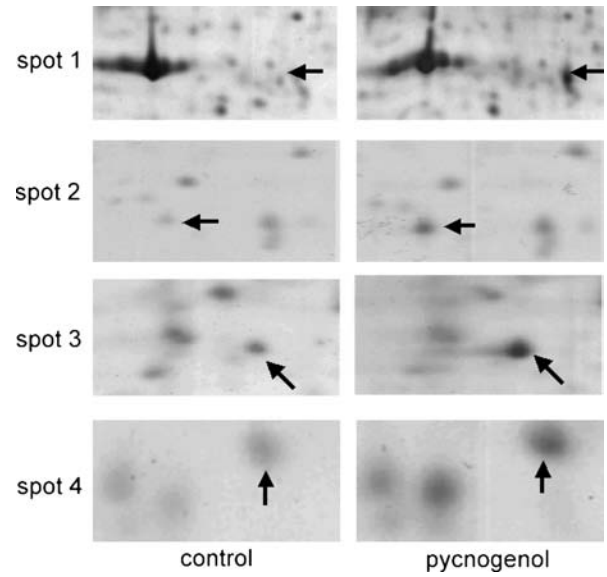

Figure 2. Pycnogenol treatment induces change of protein expression. Selected area of the spots showing intensity difference between control and pycnogenol-treated samples were amplified and indicated by arrows. These amplified images were taken from the gels identical to those shown in Figure 1.

Table 1. Pycnogenol-Induced Difference of Protein Expression Analyzed by 2D Gel Electrophoresis in RAW 264.7 Cells

	intensity (% of total volume)		fold increase	
spot no.	control	pycnogenol	(pycnogenol/control)	*P* value
1	0.10 ± 0.02	0.75 ± 0.15	7.5	<0.01
2	0.05 ± 0.01	0.43 ± 0.07	8.6	<0.01
3	0.08 ± 0.03	0.23 ± 0.07	3.5	<0.05
4	0.10 ± 0.03	0.18 ± 0.05	1.8	<0.05

The intensity of differentially expressed spots was normalized by 2D imaging software PDQuest as % of total volume, which corresponds to the intensity of each spot divided by the sum of intensities of all the spots detected in the gel by silver staining. Values are mean ± SE from eight pairs of gels.

after separation by 2D electrophoresis and visualization by silver staining. Imaging analysis indicated that there were about 700 spots detected in gels shown in **Figure 1**. Although the profiles were similar, computer analysis revealed differential protein expression between control and pycnogenol-treated samples and four upregulated spots were observed (indicated by S1–S4 in **Figure 1**) after comparison of eight pairs of gels. The selected areas in the gel containing each differentially expressed spot were amplified in **Figure 2**. The intensity and fold of differential expression of these spots was summarized in **Table 1**. Of the four spots, the occurrence of spots 1 and 2 were greatly enhanced to a level about 8-fold after overnight treatment with pycnogenol.

Identification of Differentially Expressed Proteins Induced by Pycnogenol. After establishing and mapping the proteome, it was our attempt to identify the spots whose expression is altered after pycnogenol treatment. The spots were excised, digested with trypsin, and analyzed by tandem mass spectrometry as described in Materials and Methods. The results of mass spectrometric analysis were summarized in **Table 2**. Two spots that are prominently upregulated in pycnogenol samples, spots 1 and 2, were identified as cathepsin D and keratinocyte lipid-binding protein, respectively. The identity of spot 3 was determined as annexin IV, and the last spot was proteasome

Table 2. Identification of Pycnogenol-Induced Differentially Expressed Proteins by Tandem Mass Spectrometry

spot no.	identity	protein coverage (%)	pI/molecular mass (kDa) experimental	pI/molecular mass (kDa) theoretical	SWISS-PROT accession no.
1	cathepsin D	9	5.6/43.4	6.7/44.9	P182412
2	keratinocyte lipid-binding protein	22	5.9/14.5	6.4/15	Q05816
3	annexin IV	57	5.3/36	5.4/35.9	Q7TMN7
4	proteasome subunit α type 1	35	6.3/33	6.0/29.5	Q9R1P4

subunit alpha type 1. The apparent molecular mass and isoelectric points of these proteins were comparable to the theoretical values deduced from a database (**Table 2**).

Verification of Differential Protein Expression by Immunoblotting. To confirm the pycnogenol effects detected by proteomic analysis, Western blotting was performed to examine the protein expression separated by SDS–PAGE. **Figure 3** illustrated that treatment with pycnogenol in RAW 264.7 cells enhanced expression of cathepsin D, annexin IV, and proteasome subunit alpha type 1 as compared to nontreated cells. Thus, the results from immunoblotting validate the proteomic approach in exploration of pycnogenol effects and add a list of proteins into the accumulating data of pycnogenol-regulated protein expression.

Pycnogenol-Induced Cathepsin D Expression Is Time- and Concentration-Dependent. After having explored the long-term effect of pycnogenol, the next series of experiments were conducted to investigate the time-dependent manner of pycnogenol-induced protein expression. As cathepsin D was greatly induced, we therefore analyzed its expression in cells treated for various times with pycnogenol. **Figure 4A** showed that pycnogenol at 100 μg/mL had no significant effect on the expression of cathepsin D in cells treated for up to 6 h. The inducible effect was detectable as early as 12 h after treatment and further extended to a higher level with 16 and 24 h incubation. In addition, the effect of pycnogenol displayed a concentration-dependent manner (**Figure 4B**). A small but significant effect was noted at 20 μg/mL of pycnogenol, and the induction was easily detected at 50 and 100 μg/mL.

Antioxidants and EGCG Did Not Mimic Pycnogenol Effect on Cathepsin D Expression. The findings presented above not only indicated the novel effects of pycnogenol but also raised the question of how pycnogenol mediates its effect. Pycnogenol has been shown to inhibit free radicals formation in RAW 264.7 cells (*6*–*8*). To study whether pycnogenol

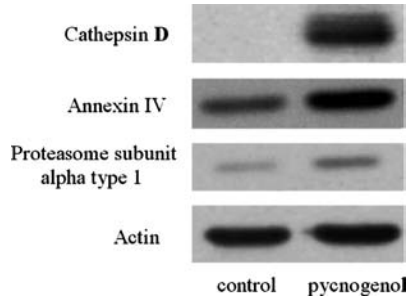

Figure 3. Verification of pycnogenol-induced protein expression by immunoblotting. Total lysates from cells incubated for 24 h in the presence or the absence of pycnogenol (100 μg/mL) were separated by SDS–PAGE, and protein expression was analyzed by Western blotting. These blots are representative of three independent experiments.

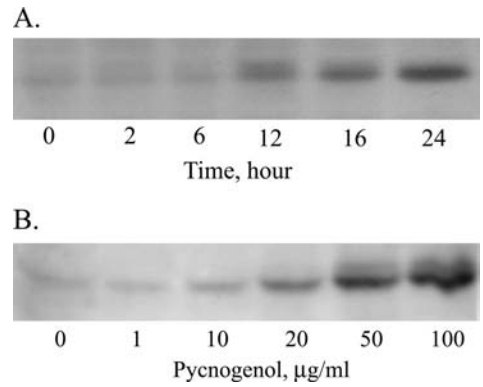

Figure 4. Temporal pattern and concentration–effect relationship of the pycnogenol effect on induction of cathepsin D. RAW 264.7 cells were treated with or without 100 μg/mL pycnogenol for various times ranging from 2 to 24 h (**A**) or incubated with various concentrations of pycnogenol for 24 h (**B**) and cathepsin D expression was determined by Western blotting. The blots are representative of three experiments.

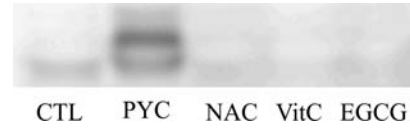

Figure 5. Pycnogenol-induced cathepsin D expression is not mimicked by antioxidants and EGCG. Cells were treated with pycnogenol (PYC, 100 μg/mL), N-acetyl cysteine (NAC, 20 mM), vitamin C (VitC, 1 mM), or epigalloyl catechin gallate (EGCG, 50 μM) for 24 h and analyzed for cathepsin D expression. A representative blot of three experiments is shown.

mediates its effect through antioxidant activity, the effect of antioxidants on cathepsin D expression was tested. As shown in **Figure 5**, pycnogenol effect was not mimicked by N-acetyl cysteine and vitamin C, suggesting that antioxidant activity is unlikely to be an important part of cathepsin D induction by pycnogenol. Because pycnogenol is composed of monomeric and oligomeric catechins (*3*, *4*), we also determined the effect of epigalloyl catechin gallate (EGCG), a monomeric catechin with similar basic structure. Unlike pycnogenol, EGCG had no effect on cathepsin D expression (**Figure 5**), indicating that pycnogenol does not mediate its effect through monomeric catechins. A recent study documented by Park et al. also showed that TNF-α secretion is stimulated by pycnogenol and procyanidin C2 (trimeric catechins) but not by monomeric and dimeric polyphenols (*8*). These phenomena are consistent with the chromatographic analysis which demonstrated that pycnogenol is primarily composed of oligomeric catechins (*3*, *4*).

Phagocytosis Is Enhanced in Pycnogenol-Treated RAW 264.7 Cells. Phagocytosis is a cellular defense mechanism that is mainly initiated by interaction of membrane receptors in phagocytes with pathogens or large particles and then internalized into a vesicular compartment called phagosome. The phagosome and lysosome are functionally associated with phagocytosis and play an important role in degradation of internalized pathogens and particles. Previous studies on cathepsin D indicate that it is a protease located in phagosome and lysosome (*13*, *14*). To further explore the effect of pycnogenol in a functional perspective after proteomic analysis, it is reasonable to postulate that pycnogenol may regulate the phagocytic activity in macrophage. In this regard, fluorescent latex beads were utilized to test the hypothesis. Results shown in **Figure 6A** clearly indicated that pycnogenol treatment leads to an increase of phagocytic activity in RAW 264.7 cells, as evidenced by the red fluorescent dots observed inside the

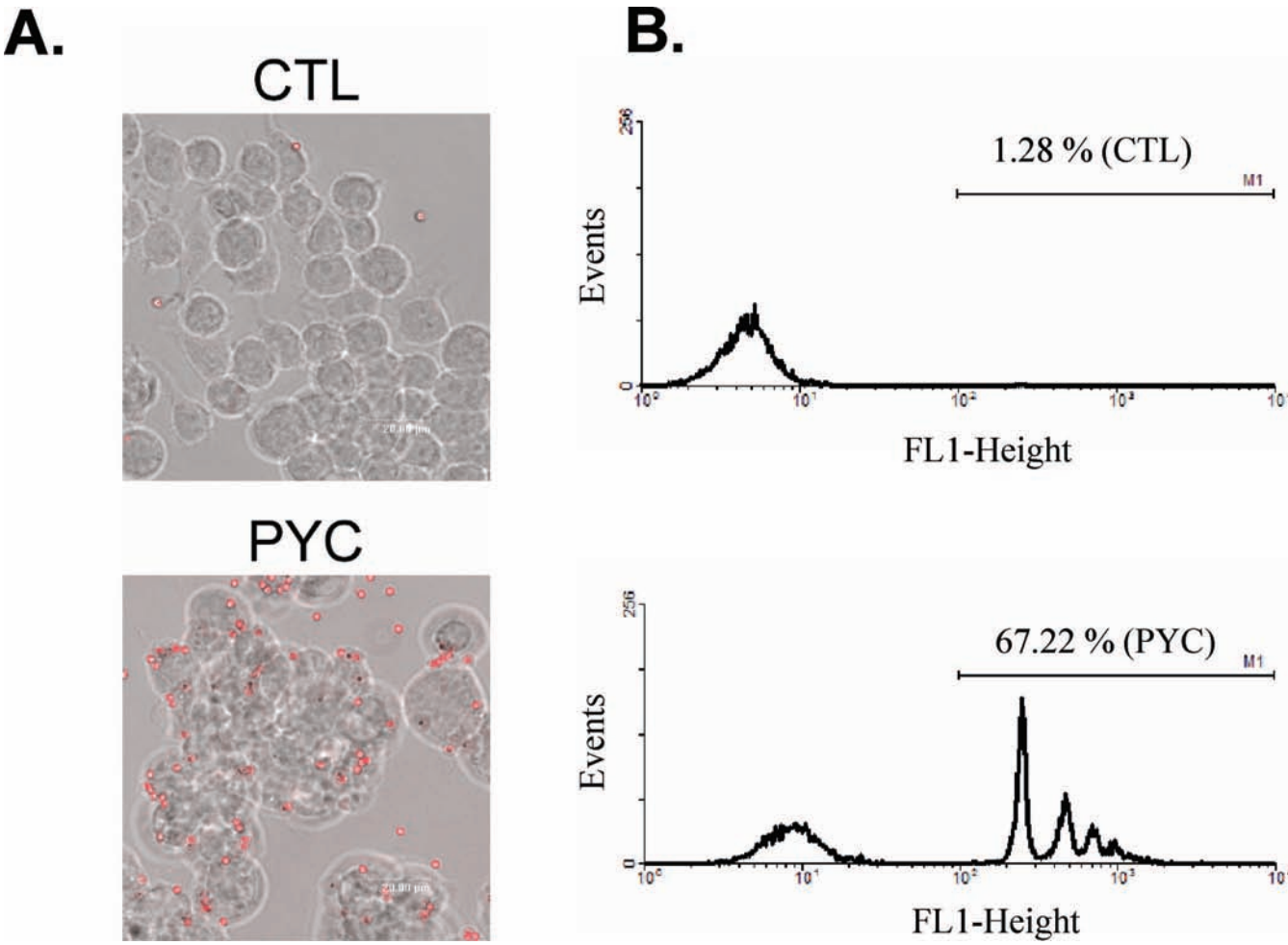

Figure 6. Pycnogenol enhances phagocytosis in RAW 264.7 cells. Cells were treated with or without pycnogenol overnight and incubated with fluorescent latex beads for 2 h. Phagocytic activity was then examined by confocal microscopy (**A**) and flow cytometry (**B**). Data shown are representative of three similar experiments.

pycnogenol-treated cells by microscopic analysis. This effect is further demonstrated in assay by flow cytometry (**Figure 6B**). A typical experiment shows that the fluorescent beads could be detected in over 50% of cells incubated with pycnogenol. In contrast, only a small portion of control cells exhibit fluorescence. Taken together, these results indicate that cathepsin D induction is correlated with phagocytosis enhancement in pycnogenol-treated macrophages.

Effect of Pycnogenol on the Number of Lysosomes. As phagocytic pathway is functionally linked to phagosome and lysosome, the observations presented in **Figure 6** prompted us to examine the effect of pycnogenol on the number of lysosome. To do this, cells were loaded with Lysosensor Green, a dye that emits fluorescence in acidic organelles such as lysosome, and pycnogenol effect was examined by confocal microscopy. **Figure 7** shows that there were a few stained lysosomes in resting cells, whereas the intensity was considerably enhanced by pycnogenol and this phenomenon was particularly notable in some of the treated cells. Thus, an increase of the number of lysosome is consistent with the effect of pycnogenol on phagocytosis in RAW 264.7 cells.

Lack of Effect of Pycnogenol on Cell Viability. In addition to its role in lysosome and phagocytosis, cathepsin D has been shown to be involved in cytokine-induced programmed cell death (*15*). Thus, it is possible that the upregulatory effects of pycnogenol in RAW 264.7 cells shown above could be associated with apoptosis. To test this possibility, effect of pycnogenol on cell viability was determined by MTT assay. As shown in **Figure 8**, there was no significant change of cell viability after treatment for 24 h with pycnogenol in a range of concentrations from 10 to 100 μg/mL. These results suggest that pycnogenol's effects on cathepsin D expression, phagocytic activity, and lysosomal staining are not linked to induction of cell death in RAW 264.7 cells.

DISCUSSION

To date, there are only a few studies using proteomics technology to document the effects of polyphenols. In a study by Deshane et al. (*16*), the effect of grape seed extract on protein expression was investigated by 2D electrophoresis and mass spectrometry, and the results suggest the potential mechanisms of its protective effect on neurodegeneration. Proteomic analysis of the extract from *Scutellaria barbata* also provides a molecular basis for its inhibitory effect on colon cancer cells (*17*). In the present study, we have employed proteomic strategy to study the inducible effect of pycnogenol in RAW 264.7 macrophage and upregulation of cathepsin D and other proteins clearly indicated a modulatory role of pycnogenol on protein expression. In contrast to previous studies on pycnogenol, these results were consistent with the enhancement role of pycnogenol on TNF-α secretion (*8*), but the inducible effect on cathepsin D required a longer time treatment (at least 12 h). In addition to its stimulatory effect, pycnogenol could be inhibitory on gene

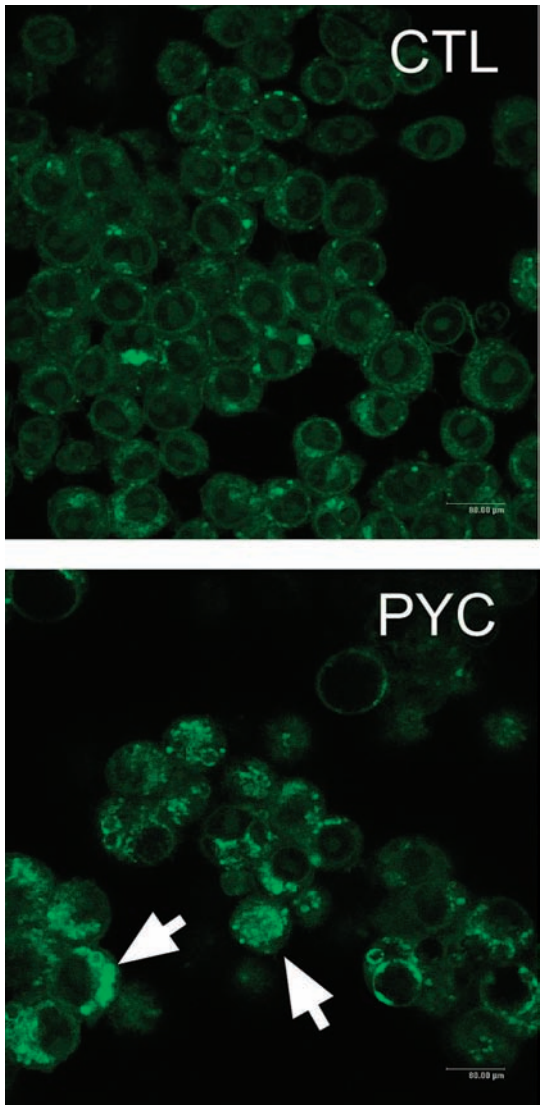

Figure 7. Effect of pycnogenol on lysosomal staining. The pycnogenol-treated (PYC) and control (CTL) cells were stained with LysoSensor Green dye and analyzed by confocal microscopy. Note the intensely stained cells indicated by arrows in pycnogenol-treated cells. The images are representative of three experiments.

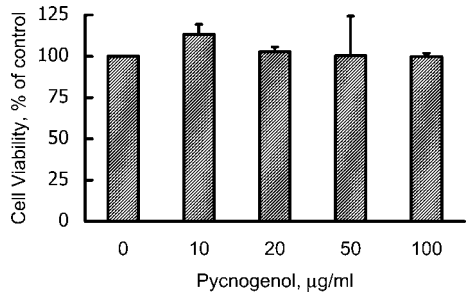

Figure 8. Effect of pycnogenol on cell viability. RAW 264.7 cells seeded in 96-well plate were treated with or without a variety of concentrations of pycnogenol as indicated for 24 h and cell viability was determined by MTT assay. The viability of cells not incubated with pycnogenol (control) was set as 100%, and the effects of pycnogenol were normalized as % of control. The results were from three independent experiments.

expression as well. This is evidenced by the studies on interleukin-1 and inducible form of nitric oxide synthase in lipopolysaccharide-treated macrophage (*6, 7*) and intercellular cell adhesion molecule-1 in TNF-stimulated endothelial cells (*10*). The concentration-effect relationship of pycnogenol-induced cathepsin D expression is also comparable to other biological effects, such as antioxidant activity, nuclear factor κB activation, TNF-α secretion, and expression of adhesion molecules (*6, 8, 10, 11*). Collectively, these findings are in agreement with the notion that pycnogenol mediates its effects through diverse pathways and it is capable of regulating protein expression in a higher or lower fashion and in resting as well as activated cells. The current study also highlights the power of proteomics-based strategy in exploration of the novel molecular targets of pycnogenol.

In addition to its regulatory effect on protein expression, we also determined the functional significance of pycnogenol implicated by the induced proteins identified through the proteomic analysis. Cathepsin D is an aspartyl protease located in the lysosome and phagosome, where it involves protein degradation and presentation of antigen after phagocytosis (*18–20*). Transgenic studies indicated that it is also involved in the development of intestinal mucosa and cells of lymphoid lineage (*21*). This protease is implicated in Batten disease, an inherited neurodegeneration disease characterized by lysosomal storage disorder (*22*). It is of interest to note that cathepsin D is overexpressed in several types of cancer and serves as a prognostic factor in breast cancer (*23*). Cathepsin D has been shown to play a role in apoptosis (*15*), although this is unlikely to be the case in this study because pycnogenol has no effect on cell viability (**Figure 8**). Annexin IV, another upregulated protein found in the present study, has been shown to localize in phagosome and is involved in antigen endocytosis (*24, 25*). Thus upregulation of cathepsin D and annexin IV by pycnogenol may be associated with changes in lysosome/phagosome and by implication in the phagocytic function of macrophage. Indeed, the results obtained from proteomics and cellular function analyses in RAW 264.7 cells are consistent with this notion and supported by the findings that pycnogenol induces cathepsin D expression and enhances phagocytosis and lysosomal staining. The functional significance of pycnogenol effects on keratinocyte lipid-binding protein and proteasome subunit α type 1 will remain to be elucidated.

Given that macrophage is a key player in immune response, the positive regulatory effect of pycnogenol observed in the present studies may suggest a better protective effect for immunity. It has been documented that the phagocytic activity of macrophages from the prematurely and chronologically aging mice models was reduced (*26, 27*) and intake of polyphenol-rich cereals led to an improvement of macrophage phagocytosis (*26*). Enhancement of phagocytosis by resveratrol, a polyphenol from grape, has been reported as well (*28*). Together these findings indicate that supplementation of polyphenols may restore phagocytic efficiency of macrophage and produce a better defense against pathogenic infection in senescent conditions. Through identification of molecular targets and elucidation of the functional significance of polyphenols treatment, these studies may also provide a scientific basis for the in vivo beneficial effects and lead to development of novel nutritional supplements.

A question raised by the observations documented in this study is how pycnogenol causes the alteration of protein expression. As it exhibits strong antioxidant activity, one obvious possibility is that pycnogenol mediates its effect via change of redox status. However, results presented in **Figure 5** do not support this possibility since compounds with potent antioxidant activity tested in this study (N-acetyl cysteine, vitamin C, and EGCG) were ineffective to upregulate cathepsin D expression.

A previous report by Tedeschi et al. also showed that the effect of green tea extract on the expression of inducible nitric oxide synthase in A549 cells is not mediated by antioxidant mechanism (*29*). These observations demonstrate that phytochemicals are able to initiate their effects through pathways distinct from the well-studied free radical scavenging activity. Elucidation of the mechanisms affected by pycnogenol is an important subject in future studies and will help gain more insight into how pycnogenol effect is achieved, but it is not clear which pathways can be accounted for.

ABBREVIATIONS USED

2D, two-dimensional; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; IPG, immobilized pH gradient; TNF-α, tumor necrosis factor-α; EGCG, epigalloyl catechin gallate; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide.

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