Basic Science

The Translational Proteome Modulated by 20(S)-Protopanaxadiol in Endothelial Cells

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Background: 20(S)-protopanaxadiol (PPD), a natural compound of dammarane ginsenoside purified from the ginseng plant, exhibits strong anticancer properties. It has also been reported to have strong antioxidant activity and plays a role in cardiovascular protection. However, the downstream signaling mechanism PPD employs is still unclear and requires further elucidation.

Methods: Endothelial cells (ECs) EAhy 926 were used to investigate the growth promoting effect of PPD. The protein lysates extracted from both mock- and PPD-treated cells were separated by two-dimensional gel electrophoresis (2-DE) to monitor protein changes. After image analysis, proteins with significant change in the expression level were further identified by mass spectrometry. Western blot was applied to further confirm the protein variations in the 2-DE assay.

Results: In the current study, we found that treatment with PPD (10 μg/ml) significantly increased ECs healing. The translational proteome was established according to 16 up-regulated and 8 down-regulated proteins identified in 2-DE. These proteins were reported to function as energy homeostasis and in the prevention of oxidative stress. The elevated expressions of heme oxygenase 1 (HO-1) and glutathione synthetase (GSS) were further confirmed in the western blot analysis.

Conclusions: According to the information obtained from translational proteome, we delineated that PPD mediated vascular homeostasis through the up-regulation of anti-oxidative proteins. Additional functional investigations are necessary regarding the HO-1 and GSS proteins.

Key Words: Dammarane sapogenins • Endothelial cell • Glutathione synthetase • Heme oxygenase 1 • Proteome • 20(S)-protopanaxadiol

INTRODUCTION

Ginseng is an ancient herb and has a long history of use as traditional medicine in Asian countries.[1] With the advances in modern biotechnology, an active fraction from ginseng plant that contains more than 25 dammarane-type ginsenosides has been isolated, and the large majority of them exhibit anticancer properties.[2] These compounds also show preventive effects in diabetes, neuronal degeneration and inflammation.[3,4] Having a preventive effect in cardiovascular diseases is regarded as an important therapeutic effect of ginseng. Treatment with ginsenoside can modulate cellular calcium level and therefore contributed to a vasorelaxing effect in the coronary artery.[5] Two ginsenosides, protopanaxadiol and protopanaxatriol could modulate

Received: September 18, 2013 Accepted: January 20, 2014

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endothelial cell (EC) functions through both the glucocorticoid receptor and oestrogen receptor.[6] In patients with coronary artery disease, Korean red ginseng has been observed to reduce vascular stiffness.[7] By inhibiting the JAK/STAT pathway, ginseng inhibits smooth muscle cell proliferation that reduces diverse vascular diseases.[8] Saponin extracted form *Panax notoginseng* could protect vascular via up-regulating nitric oxide biosynthesis, a gaseous molecule with a significant role in endothelial integrity.[9] Madecassoside, a triterpenoid saponin purified from *Centella asiatica* could protect ECs from H_2O_2-induced oxidative injury.[10] All of these examples indicated that ginsenosides are important in maintaining cardiovascular function. However, most of these studies were focused on the expression of particular proteins instead of a global observation of proteome change. Therefore, the signaling cascade still remains unclear.

20(S)-protopanaxadiol (PPD) is one of the most studied aglycones of those ginsenosides extracted from *Panax quinquefolius*. In the vascular system, PPD has exhibited a vasorelaxing effect and maintained vascular integrity.[5,6] PPD can reduce hyperglycemia-induced risk factor in patients.[10] Pretreatment of PPD prior to H_2O_2 exposure could increase cell viability of neonatal rat cardiomycocytes coupled with increased activity of superoxide dismutase (SOD) and the decreased level of lactate dehydrogenase (LDH), which indicates that PPD has a cardioprotective effect by increase cellular antioxidant enzymes.[11]

Two-dimensional gel electrophoresis (2-DE) is a feasible approach to simultaneously monitor the expression levels of a number of proteins during various physiological stimuli.[12] In the current study, we identified 16 up- and 8-down-regulated proteins. Most of them have been reported to be involved in energy production and the prevention of oxidative stress. Further western blot analysis demonstrated that PPD can enhance the expression levels of heme oxygenase 1 and glutathione synthetase. Therefore, in the PPD-promoted rapid growth ECs, the role of mitochondria and antioxidants are key factors in understanding the molecular signaling mechanism.

METHODS

Cell culture and drug treatment

The EC line EAhy 926 was generously donated by Cora-Jean S. Edgell, University of North Carolina, Chapel Hill, North Carolina, USA. ECs were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, streptomycin (100 μg/mL) and penicillin (100 U/mL) with 5% CO_2 at 37 °C. Prior to use, the ECs were incubated overnight in the same medium containing 2% fetal bovine serum. ECs were then subjected to a series of concentrations of dimethyl sulfoxide-dissolved PPD for wound healing test.

Wound healing assay

The cultured ECs were scratched using a sterilized tip and then separately incubated with a series of concentrations of PPD (10, 25 and 50 mg/L) and photographed at intervals of 0, 3 and 6 h. The percentage of wounded region in the panel was calculated using TScratch software.

Cytotoxicity assay

Around 1×10^5 cells were cultured in a microplate and then treated with a series of concentrations of PPD (1, 10, 25, 50, 100 and 200 mg/L). Before harvest, 10 μl WST-1 reagent (Roche Applied Science, Mannheim, Germany) was added to each well and incubated at 37 °C for 0.5 h under dark conditions. After shaking for 1 min, the cultured medium was measured by enzyme-linked immunosorbent assay reader with the wavelength at 450 nm.

Cell lysis and protein extraction

PPD-treated ECs were washed with a wash buffer (10 mM HEPES, pH 7.4, containing 140 mM NaCl, 4 mM KCl, and 11 mM glucose) and pelleted by centrifugation. Cell lysates were obtained by sonication in lysis buffer [250 mM HEPES, pH 7.7, containing 1 mM ethylenediaminetetraacetic acid, 0.1 mM neocuproine and 0.4% (w/v) CHAPS]. The protein concentration was determined using the BCA protein assay reagent (Thermo Fisher Scientific Inc., Rockford, IL, USA).

Two-dimensional gel electrophoresis

Extracted protein (1 mg) of PPD-treated ECs was precipitated with 3 volumes of cold acetone at -20 °C for at least 20 min. After centrifugation, the protein pellets were air-dried for 5 min, dissolved in sample buffer [9 M urea, 2% (w/v) CHAPS, 60 mM DTT, 2% (v/v, pH 4-7) IPG

buffer (GE Healthcare BioSci., NJ, USA)], and incubated for at least 30 min to denature proteins completely. The protein solution was mixed with rehydration solution [8 M urea, 2% (w/v) CHAPS and 0.5% (v/v, pH 4-7) IPG buffer] to reach a final volume of 340 μL, and then soaked into an 18 cm DryStrip (pH 4-7, GE Healthcare Life Science) for up to 12 h on Ettan IPGphor system (GE Healthcare BioScience). Iso-electric focusing (IEF) was performed with the accumulated voltage set to 32 kVh. After IEF analysis, stripped gels were equilibrated with Tris-buffer [50 mM Tris-HCl, pH 8.8, containing 2% (w/v) SDS, 6 M urea, 30% (v/v) glycerol and 60 mM DL-dithiothreitol (DTT)] for 20 min. The stripped gels were then alkylated in the same buffer containing 135 mM iodoacetic acid for additional 20 min. The equilibrated IEF strip was laid on top of a vertical sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) system to perform the 2-DE.

Image analysis and protein in-gel digestion

The gels were stained with VisPRO dye (Visual Protein Biotech., Taipei, Taiwan) and scanned with a digital scanner (Microtek International Inc.). The translational level of PPD-regulated proteins was calculated using Progenesis Samespots v2.0 software (NonLinear Dynamics, Newcastle, UK). The gel slices excised from the VisPRO-stained gel were digested with trypsin for 4 h at 37 °C (In-Gel Tryptic Digestion Kit, Thermo Fisher Scientific Inc.).

Mass spectrometric analysis

After in-gel digestion, the trypsic peptides were desalting with a Proteomics C18 Column (Mass Solution Ltd., Taipei, Taiwan) and then subjected to mass analysis using a CapLC/Q-TOF mass spectrometer (Micromass, Manchester, UK). MS data were searched against the NCBInr database using a MASCOT in-house search program (Matrixscience, London, UK). Search parameters were set as: Mass Values: Monoisotopic; Protein Mass: Unrestricted; Peptide Mass Tolerance: ± 0.4 Da. Fragment Mass Tolerance; ± 0.4 Da. Max Missed Cleavages: 1, and the instrument type: ESI-QUAD-TOF.

Western blot

Samples of the cell lysate (40 μg) were mixed with SDS-PAGE sample buffer [62.5 mM Tris-HCl, pH 6.8, 3% (w/v) SDS, 5% (v/v) 2-mercaptoethanol and 10% (v/v) glycerol], and then separated by SDS-PAGE. The blotted membranes were hybridized with monoclonal antibodies of heme oxygenase 1 (Abcam, Cambridge, UK) and glutathione synthetase (Abcam), developed with the SuperSignal West Femto reagent (Thermo Fisher Scientific Inc.), and exposed to X-ray films. The images on X-ray films from three replicates were scanned using a digital scanner (Microtek International Inc., Hsinchu, Taiwan) and were analyzed using Progenesis Samespots v2.0 software (NonLinear Dynamics) to determine the level of protein expression.

RESULTS

20(S)-protopanaxadiol promoted endothelial cell migration

Cultured ECs were subjected to dual parameters (concentration and incubation time) to investigate the motility of cell migration using a wound healing assay. The cytotoxicity of PPD was pretested from 1 mg/L to 200 mg/L. The half maximal inhibitory concentration (IC_{50}) was estimated at 100 mg/L (Figure 1A). As shown in Figures 1B and C, treatment with 10 mg/L of PPD resulted in the largest migration rate, compared with other treatments. The wounded region was significantly healed (from 42.48% to 17.67%) within 6 hours and showed an approximately 4-fold increase in migration rate as compared with control. All the treated ECs were completely healed after 16 h.

Identification of PPD-modulated proteome

ECs lysates extracted from either mock- or PPD-treated cells were analyzed by 2-DE. After VisPRO dye staining, gel images were analyzed using software. After three independent repeated 2-DE, the expression levels of 16 proteins were significantly up-regulated (fold change $\geqq$ 1.5), while expression levels of 8 proteins were significantly down-regulated (fold change $\leqq$ 0.5) (Figure 2). These proteins were analyzed by mass spectrometry and then identified by searching against the SwissProt database (Table 1). Approximately 1/3 of them are involved in energy production, including cytochrome b (GU3), pyruvate kinase (GU6), mitochondria-enoyl-CoA hydratase (GU10), 6-phosphogluconolactonase

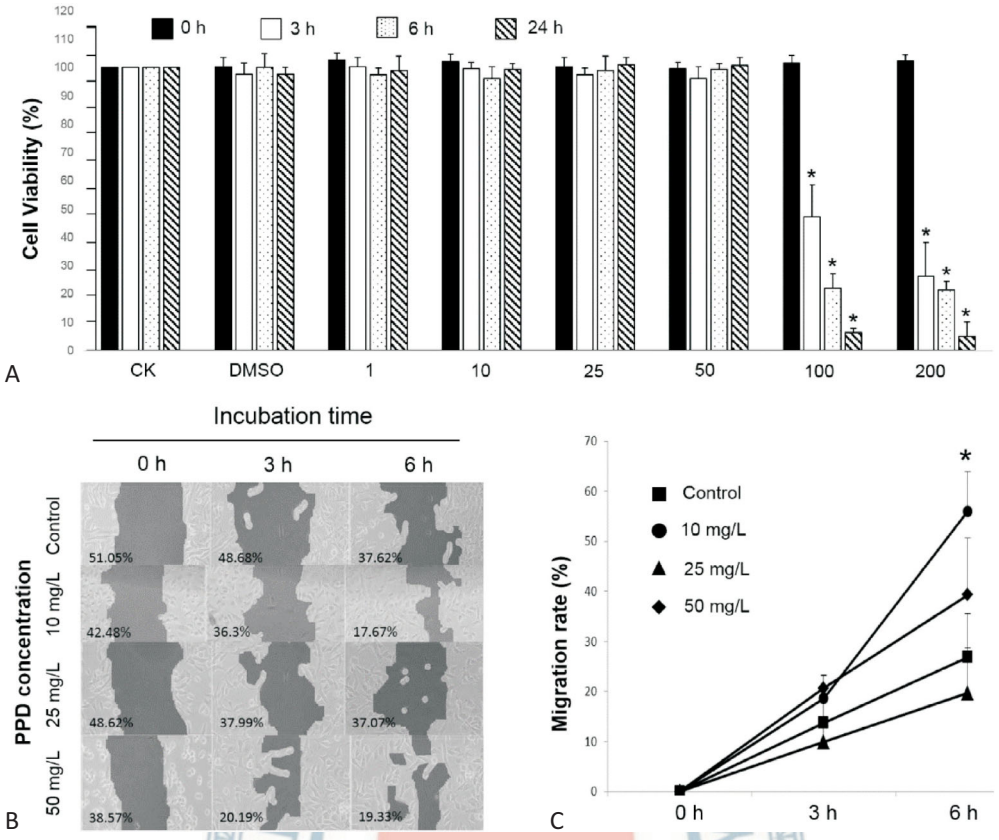

Figure 1. *20(S)-protopanaxadiol promote endothelial cell migration. (A) The cytotoxicity of PPD with different concentrations (1, 10, 25, 50, 100 and 200 mg/L) and treating times (0, 3, 6 and 24 hours) were estimated by WST-1 assay kit. (B) In wound healing assay, cultured ECs were scratched using a sterilized tip and then separately incubated with a series of concentrations of PPD (10, 25 and 50 mg/L) and photographed at times of 0, 3 and 6 h. The percentage of wounded region in the individual panel was calculated using TScratch software. (C) The percentage of wounded region was converted to a relative migration rate that was statistically calculated from 3 separate experiments. The relative migration rate is shown as the mean ± S.E. compared with control treatment. Statistical significance (* $p < 0.05$) was evaluated using Fisher's LSD. ECs, endothelial cells; PPD, 20(S)-protopanaxadiol.*

(GU11) and mitochondria-ATP synthase (GU15). There were also two proteins that function directly as antioxidants: glutathione synthetase (GU2, GSS) and glutathione transferase (GU8). A key enzyme that can confer antioxidative stress, heme oxygenase 1 (GU16, HO-1) was also induced under PPD treatment.

Heme oxygenase 1 and glutathione synthetase were up-regulated by PPD

According to the proteome shown in 2-DE, the expressions of heme oxygenase 1 (HO-1) and glutathione synthetase (GSS) were increased as compared to the control treatment. To verify the accuracy of this expression level, we used monoclonal antibody against HO-1 and GSS in a western blot analysis. As shown in Figure 3, the expression level of HO-1 dramatically increased after treatment with PPD for 3 h, and reached the maximum level around 8-fold as compared with the control treatment within 6 h. However, the HO-1 level started to decrease at 24 h. As for the level of GSS, it was increased approximately 4.5-fold at 6 h and sustained to 24 h.

DISCUSSION

20(S)-protopanaxadiol is regarded as a modern herbal medicine extracted from the traditional ginseng plant in Asian regions. With advances in plant compound extraction technology, the pure compounds are available for further medical research. In the present study, we addressed the additional effects of PPD on the vascular system, which are different than their anticancer activity.

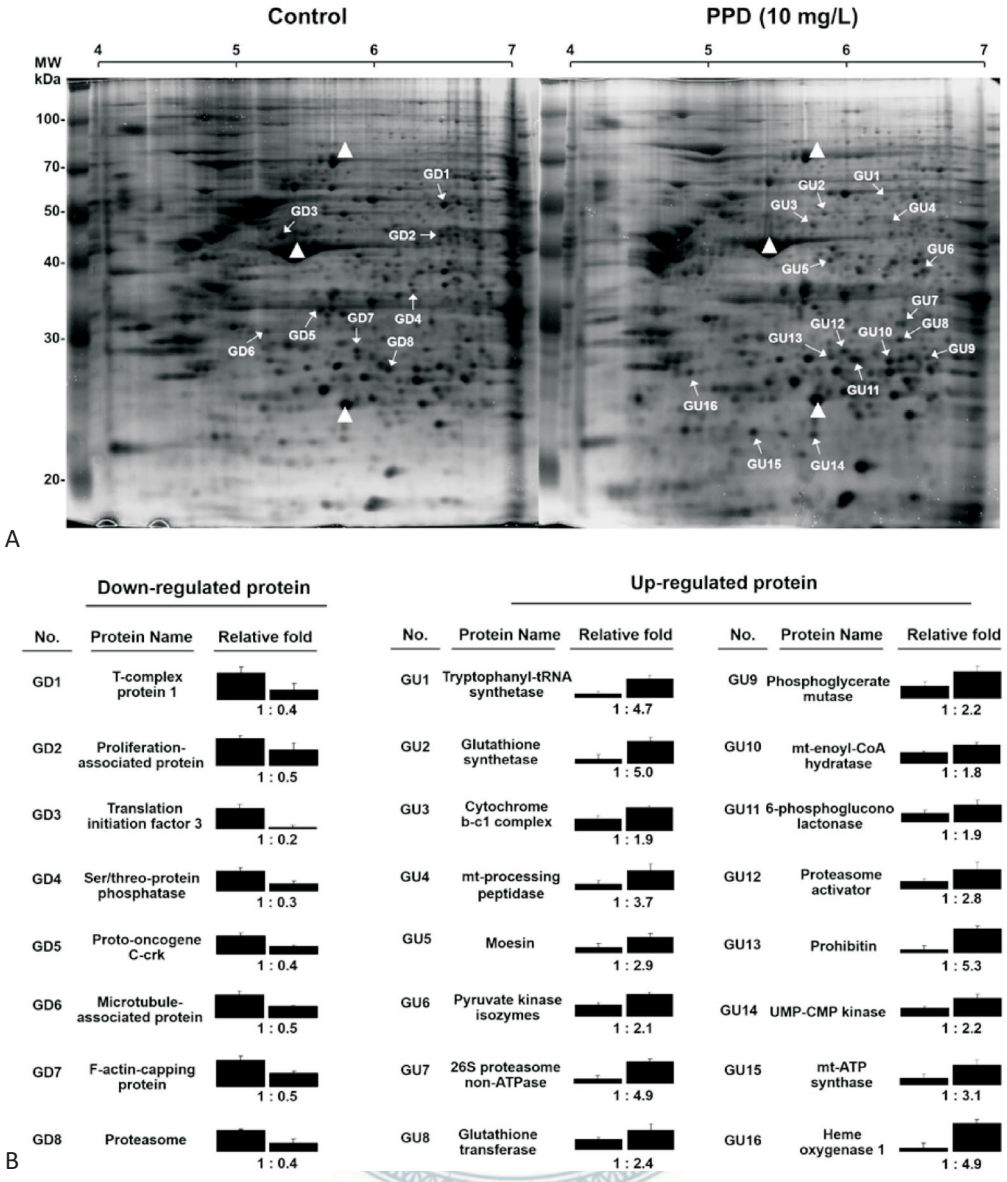

Figure 2. *The translational proteome modulated by 20(S)-protopanaxadiol. (A) Protein samples (1 mg) from mock- or PPD-treated (10 mg/L) for 6 h were subjected to 2-DE. Results were revealed by VisPRO staining. The numbers denoted on the mock treatment gel represent the proteins whose expression was down-regulated (GD1-8), while numbers denoted on the PPD treatment gel represent proteins whose expression was up-regulated (GU1-16). Solid triangles were used as reference to rectify the relative location of each protein spot. (B) The relative fold change in protein level was shown by spot density as mean ± S.E. from three separate experiments. The average ratio was also indicated under the statistic bar. The selected protein spots were picked up by pipette tip and then subjected to LC-MS-MS for protein identification. 2-DE, two-dimensional gel electrophoresis; PPD, 20(S)-protopanaxadiol.*

Proteomics, the global screening of protein variation under certain conditions, has been well-established for decades. As shown in Table 1, at least three classes of proteins were highly correlated with the EC migration phenomenon and are worthy of discussion. The mechanism of cell migration might be due to the activation of matrix metalloproteinase-2 (MMP-2) and MMP-9 that is accompanied by the enhanced activity of proteasome.[13] Although the level of proteasome (GD8) was decreased in the current study, the levels of two proteins, 26S proteasome (GU7) and proteasome activator (GU12), were found to be enhanced, suggesting that the proteasome may participate in modulating PPD-mediated cell proliferation and migration.

Table 1. Identification of 20(S)-protopanaxadiol-modulated proteins with nLC-MS/MS

Spot no.	Protein name*	Accession no.#	MW(kDa)/pI Thero.†	MW(kDa)/pI Exp.‡	Sequence coverage (%)	MOWSE Score	Peptides Matched
Up-regulated							
GU1	Tryptophanyl-tRNA synthetase	SYWC_HUMAN	53.5/5.8	53.5/6.2	10	193	4
GU2	Glutathione synthetase	GSHB_HUMAN	52.5/5.7	48.9/5.8	53	647	25
GU3	Cytochrome b – c1 complex subunit 1	QCR1_HUMAN	53.3/5.9	47.1/5.7	20	200	10
GU4	Mitochondrial processing peptidase beta-subunit	MPPB_HUMAN	55.1/6.4	47.4/6.3	17	175	8
GU5	Moesin	MOES_HUMAN	67.9/6.1	38.6/5.8	27	659	22
GU6	Pyruvate kinase isozymes M1/M2	KPYM_HUMAN	58.5/7.9	38.0/6.6	8	66	4
GU7	26S proteasome non-ATPase regulatory subunit 14	PSDE_HUMAN	34.7/6.1	35.7/5.9	6	50	2
GU8	Glutathione transferase omega-1	GSTO1_HUMAN	27.8/6.2	29.1/6.3	31	219	9
GU9	Phosphoglycerate mutase 1	PGAM1_HUMAN	28.9/6.7	27.3/6.7	24	316	6
GU10	Mitochondrial enoyl-CoA hydratase	ECHM_HUMAN	31.8/8.3	27.2/6.2	35	326	10
GU11	6-phosphogluconolactonase	6PGL_HUMAN	27.8/5.7	27.2/6.1	55	335	11
GU12	Proteasome activator complex subunit 1	PSME1_HUMAN	29.0/5.8	28.5/6.0	8	56	2
GU13	Prohibitin	PHB_HUMAN	29.8/5.6	27.3/5.9	15	130	4
GU14	UMP-CMP kinase	KCY_HUMAN	22.4/5.4	23.0/5.7	20	198	4
GU15	Mitochondrial ATP synthase subunit d	ATP5H_HUMAN	18.5/5.2	23.1/5.3	49	182	7
GU16	Heme oxygenase 1	HMOX1_HUMAN	32.8/4.8	26.0/4.8	21	165	3
Down-regulated							
GD1	T-complex protein 1 subunit beta	TCPB_HUMAN	57.8/6.0	54.5/6.5	36	455	19
GD2	Proliferation-associated protein 2G4	PA2G4_HUMAN	43.8/6.1	47.4/6.4	8	69	3
GD3	Eukaryotic translation initiation factor 3 subunit F	EIF3F_HUMAN	37.5/5.2	45.9/5.3	10	126	3
GD4	Serine/threonine-protein phosphatase PP1-alpha catalytic subunit	PP1A_HUMAN	38.2/5.9	37.5/6.3	3	46	2
GD5	Proto-oncogene C-crk	CRK_HUMAN	33.9/5.4	34.2/5.7	12	105	3
GD6	Microtubule-associated protein RP/EB family member 1	MARE1_HUMAN	30.0/5.0	31.4/5.2	7	53	1
GD7	F-actin-capping protein subunit beta	CAPZB_HUMAN	31.6/5.4	29.1/5.9	15	152	5
GD8	Proteasome subunit beta type-7	PSB7_HUMAN	30.0/7.6	27.2/6.2	16	160	4

* Function of the protein obtained via the MASCOT software (www.matrixscience.com) search program by querying the SwissProt database. The parameters were set at peptide mass tolerance: ±0.4 Da; allowed missed cleavage: 1. # Accession number from SwissProt database. † Theoretic protein molecular weight and pI annotated in SwissProt database. ‡ Experimental protein molecular weight and pI calculated from 2-DE gel.

2-DE, two-dimensional gel electrophoresis; MW, molecular weight.

Cell aging has recently been regarded as the most critical factor in pathogenesis. This process is highly related to mitochondrial integrity and energy demands.[14,15] The previous study suggested that (20S)-ginsenoside prevents EC apoptosis via Akt-dependent inhibition of the mitochondrial apoptotic signaling pathway.[16] In our profiling of PPD-enhanced proteins, we found that approximately 1/3 of those proteins functioned in energy production, including cytochome b, pyruvate kinase, mitochondria-enoyl-CoA hydratase, 6-phosphogluconolactonase and mitochondria-adenosine triphosphate synthase. These indicates that energy yield through mitochondrial integrity is an essential contribution of PPD towards ECs proliferation and vascular homeostasis, and this is valuable for developing new pharmaceutical means that will control unwanted endothelial cell death at the site of vascular injury.

Oxidative stress is the most serious factor in the induction of cellular dysfunctions and pathologies. The production of reactive oxygen species, including free

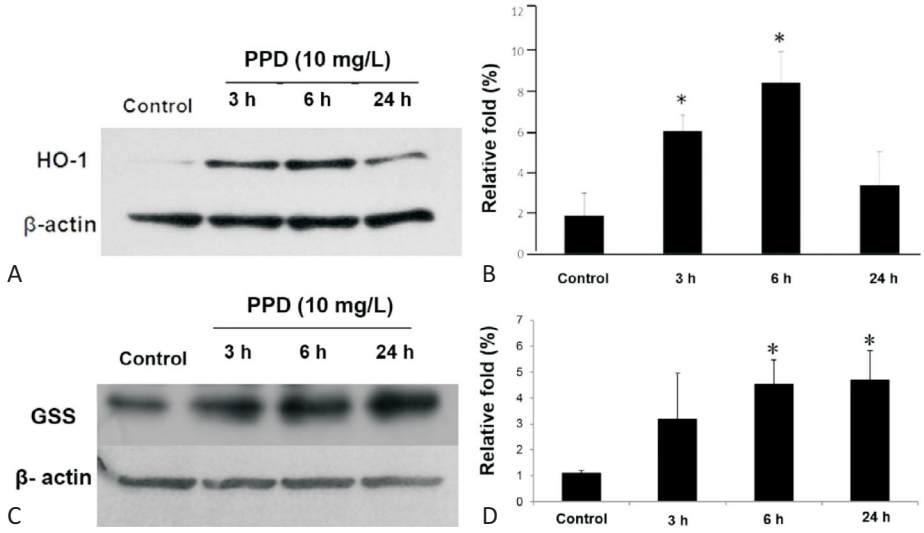

Figure 3. *20(S)-protopanaxadiol enhanced the expressions of heme oxygenase 1 and glutathione synthetase. (A,C) ECs were treated with PPD (10 mg/L) for 3, 6 and 24 h. Cell lysates (40 μg) were separated by SDS-PAGE and transferred to a membranes that were probed with and anti-heme oxygenase 1 (HO-1) monoclonal antibody (1:1000) and probed with an anti-glutathione synthetase (GSS) monoclonal antibody (1:2000). (B,D) The increase in HO-1 and GSS expressions, relative to the control, were calculated from 3 separate experiments. Values are shown as the mean ± S.E., compared to control treatment. Statistical significance (* $p < 0.05$) was evaluated using Fisher's LSD. GSS, glutathione synthetase; PPD, 20(S)-protopanaxadiol.*

radicals and peroxides, is a particularly destructive aspect of oxidative stress.[18] HO-1 is an antioxidant, anti-inflammatory, and cytoprotective enzyme that is induced in response to oxidative stress.[19] It was also functionally highlighted as a molecular switch in wound healing.[20] Glutathione has antioxidant properties in the whole cell because the thiol group in its cysteine moiety is a reducing agent which can be regenerated after its oxidation.[21,22] In our study, PPD was found to increase the expression of these three antioxidant enzymes, HO-1, GSS and glutathione transferase that are responsible for detoxification reactions. This finding suggests that PPD can protect ECs from oxidative stress generated during the rapid wound-healing process.

Therefore, we can conclude that the function of sapogenins in vascular homeostasis may be through its effects on antioxidative effect during energy production, although the redox status merits further study.

ACKNOWLEDGEMENTS

This work was supported, in part, by grants from the National Science Council (NSC 101-2320-B-037-041), the Kaohsiung Medical University Research Foundation (KMU-Q110003), and the Chi-Mei Medical Center and Kaohsiung Medical University Research Foundation (101CM-KMU-01). We are grateful to the core facility laboratory of the Institute of Biomedical Sciences, Academia Sinica, and the Center for Resources, Research & Development of Kaohsiung Medical School for the mass spectrometric analysis. We also acknowledge Pegasus Pharmaceuticals Group, Canada, for providing the 20(S)-protopanaxadiol powder.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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