Glossogyne tenuifolia Enhances Posttranslational S-Nitrosylation of Proteins in Vascular Endothelial Cells

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ABSTRACT: *Glossogyne tenuifolia* (GT) is a traditional Chinese herb that possesses strong antioxidant activity and protects against endothelial cell (EC) injury by inhibition of free reactive oxygen species (ROS). The aim of this study was to elucidate the mechanisms by which GT prevents endothelial injury using a proteomics approach. We used a sensitive method to analyze the S-nitrosoproteins utilizing a modified biotin-switch method in order to detect the possible effects of GT on protein posttranslational modification. After treatment of vascular ECs with GT, two proteins HspA9 (IS1), beta-actin (IS2) were observed to have increased posttranslational S-nitrosylation, whereas seven proteins, vimentin (DS2, DS3 and DS5), tropomyosin 3, 4 (DS6 and DS7) and oxidative phosphorylation protein such as ATP synthase, F1 complex (DS1) and 80K-H protein (DS4), were found to have decreased posttranslational S-nitrosylation. Due to S-nitrosylation of HspA9 causing the reduction of intracellular ROS and S-nitrosylation of ATP synthase interfering with ATP production and ROS formation, our study may indicate a novel mechanism in which GT protects EC injury by the inhibition of oxidative reaction.

KEY WORDS: Antioxidation, free reactive oxygen species, *Glossogyne tenuifolia*, posttranslational S-nitrosylation of proteins, vascular endothelial cells.

INTRODUCTION

Glossogyne tenuifolia (GT) is a traditional Chinese herb that is mainly available in Southern Asia, Australia, New Caledonia and southern Taiwan. The effective components of GT identified include luteolin, luteolin-7-glucoside and oleanolic acid (Hsu et al., 2005; Wu et al., 2005). Recent studies have shown that the major effects of GT are as follows: (1) it is cytotoxic to human hepatocellular carcinoma cells and inhibits hepatitis B virus replication; (2) it promotes the anti-oxidation of tumour necrosis factor-alpha (TNF-α), interleukin (IL)-6 and interferon-gamma (IFN-γ) in human whole blood and may prevent atherosclerosis by inhibiting the oxidation of low-density lipoproteins (LDLs) and the production of reactive oxygen species (ROS) in human leukocytes; and (3) it acts as an immunomodulator by inhibiting the synthesis of proinflammatory mediators in activated murine peritoneal macrophages and splenocytes via the NF-κB dependent pathway (Hsu et al., 2005; Wu et al., 2005; Yang et al., 2006; Ha et al., 2006).

The released NO mediates its effects via guanylate cyclase to increase the level of cGMP in the cardiovascular system and thereby results in vasodilatation, anti-thrombosis and anti-proliferation (Murad, 1999; Yang et al., 2006). Previous studies have shown that NO may directly react with cysteine residues so that the latter undergo S-nitrosylation – a generic protein modification that is required for the regulation of diverse protein functions and signalling mechanisms (Ravi et al., 2004). The aim of this study is to elucidate the mechanism of action of GT by using a recently modified method (Huang et al., 2009) to improve the detection sensitivity of S-nitrosoproteins and to examine the possible reactions involved in the prevention of endothelial injury.

MATERIALS AND METHODS

Plant material and preparation of the ethanol extract of GT

The raw materials for GT were bought from an herbal store in Penghu Island, and then later confirmed by using DNA sequence identification (Hsu et al., 2005). Dried whole plant materials of GT (5.3 kg) were crushed and soaked in 20 L ethanol for 1 day and then extracted 3 times with the same volume of ethanol.

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After filtration through medical gauze, the filtrate was collected and concentrated in a vacuum evaporator. The weight of the crude extract was 777 g, and the yield was 14.7%.

Cell culture and chemical treatment

The endothelial hybridoma (EAhy926) cell line was used and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS), streptomycin (100 μg/ml) and penicillin (100 U/ml) at 37°C under 5% CO_2. After overnight starvation in 2% FBS, the ECs were exposed to a series of dimethyl sulfoxide (DMSO) solutions containing the crude extract of GT; the concentrations of the solutions were 1, 5, 10 and 20 μg/ml. A previous study revealed the time and dose dependent effects of GT on murine macrophage cell and human whole blood (Wu et al., 2005). In our study, we demonstrated that the endothelial cells (ECs) were incubated for 30 min, 1 h or 6 h in order to determine the expression of S-nitrosylation protein.

Two-dimensional gel electrophoresis

After the ECs were exposed to GT, they were washed with cold buffer (0.14 M NaCl, 4 mM KCl, 11 mM glucose, 10 mM 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid [HEPES], pH 7.4). Whole cell lysates were obtained by sonication in isoelectric focusing (IEF) sample buffer (9 M urea, 4% w/v 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate [CHAPS], 1% w/v dithiothreitol [DTT] and 2% v/v immobilized pH gradient [IPG] buffer [pH 4-7]). Proteins (1 mg) were mixed in a rehydration solution (8 M urea, 2 % w/v CHAPS, 1% w/v DTT and 0.5 % v/v IPG buffer [pH 4-7]) to attain a final volume of 340 μl, and an 18-cm DryStrip (pH 4-7) was then soaked in this solution for up to 12 h using the Ettan IPGphor. The voltage was maintained at 40 kV/h. After IEF analysis, the stripped gels were equilibrated with 2% (w/v) sodium dodecyl sulfate (SDS), 50 mM Tris-HCl (pH 8.8), 6 M urea and 30% (v/v) glycerol. The equilibrated IEF gels were loaded on top of a vertical SDS-polyacrylamide gel electrophoresis (PAGE) system.

To determine the specificity of biotin-HPDP after S-nitrosocysteine labelling, aliquots (10μg/ml) of whole cell lysate (WCL), MMTS-blocked lysate and ascorbate-reduced lysate (As) were collected at each step of the biotin-switch procedure. A negative control was prepared by adding 1% (w/v) DTT (As+DTT). Each reactant (40μg) was separated using 10% SDS-PAGE and subjected to Western blotting with streptavidin-HRP (1:4000) prior to development on X-ray films.

Biotin switch, Western blotting and image analysis

The S-nitrosoproteins were biotinylated according to the biotin-switch method that was developed by Jaffrey and Snyder (Jaffrey et al., 2001). In contrast to standard 2-dimensional electrophoresis (2-DE), DTT and tris (2-carboxyethyl) phosphine (TCEP) were not used in any of the above mentioned processes (Huang et al., 2009). After the gels were separated by 2-DE, they were stained with VisPRO (Visual Protein Biotech., Taipei, Taiwan) for 10 min and scanned with the UMAX Astra 1200S scanner. The gels were destained with SDS-PAGE running buffer for 2 min and then blotted onto a nitrocellulose membrane. After the membrane was blocked with non-fat dried milk (5% w/v) for 2 h, it was hybridized with 1:4000 streptavidin-conjugated horseradish peroxidase (HRP) (PerkinElmer LifeSci., MA, USA) for 2 h. The membrane was then washed with Tris-Tween-buffered saline (TTBS) (20 mM Tris-HCl [pH 7.5], 500 mM NaCl and 0.075% v/v Tween-20) and was exposed on X-ray films using SuperSignal West Femto Maximum chemiluminescence reagent (Pierce Biotech). The spots with densities that indicated posttranslational S-nitrosylation were scanned with the UMAX Astra 1200S scanner and then analyzed with the ImageMaster software. A total of 3 standard protein bands on the gel images and X-ray films were established to facilitate the identification of proteins from other gels. All the experiments were repeated three times.

In-gel digestion and mass spectrometric analysis

The S-nitrosoprotein spot densities that were significantly increased or decreased (>1.5 fold or <0.7) after GT could be identified accurately. The gel slices were digested at 37°C for 4 h using the In-Gel Tryptic Digestion Kit (Pierce BioSci.). The tryptic peptides were desalted using a protein C18 column (C Sun Mfg. Taipei, Taiwan) and mass analyzed using CapLC-Q-TOF (Micromass, Manchester, UK). The mass spectrometry (MS) data was searched against the NCBInr database using the in-house MASCOT search program (Matrixscience, London, UK). The sites of biotinylated cysteine residues, i.e., the S-nitrosylation sites, were screened on the basis of a mass shift of 428.2 Da. The relative ratios of biotinylation between the GT and control treatments were estimated using Tandem MS/MS Automated Selected Ion Chromatogram (MASIC) software.

Analysis of posttranslationally modified S-nitrosoproteins

Whole cell lysate was subjected to three steps of the biotin-switch process: methyl methanethiolsulfonate (MMTS)- blocking, ascorbate reduction and biotin-HPDP substitution. On Western blotting, the whole cell lysates that contained very high levels of free cysteine thiols showed the highest biotinylation. Biotinylation was strongly inhibited after these free

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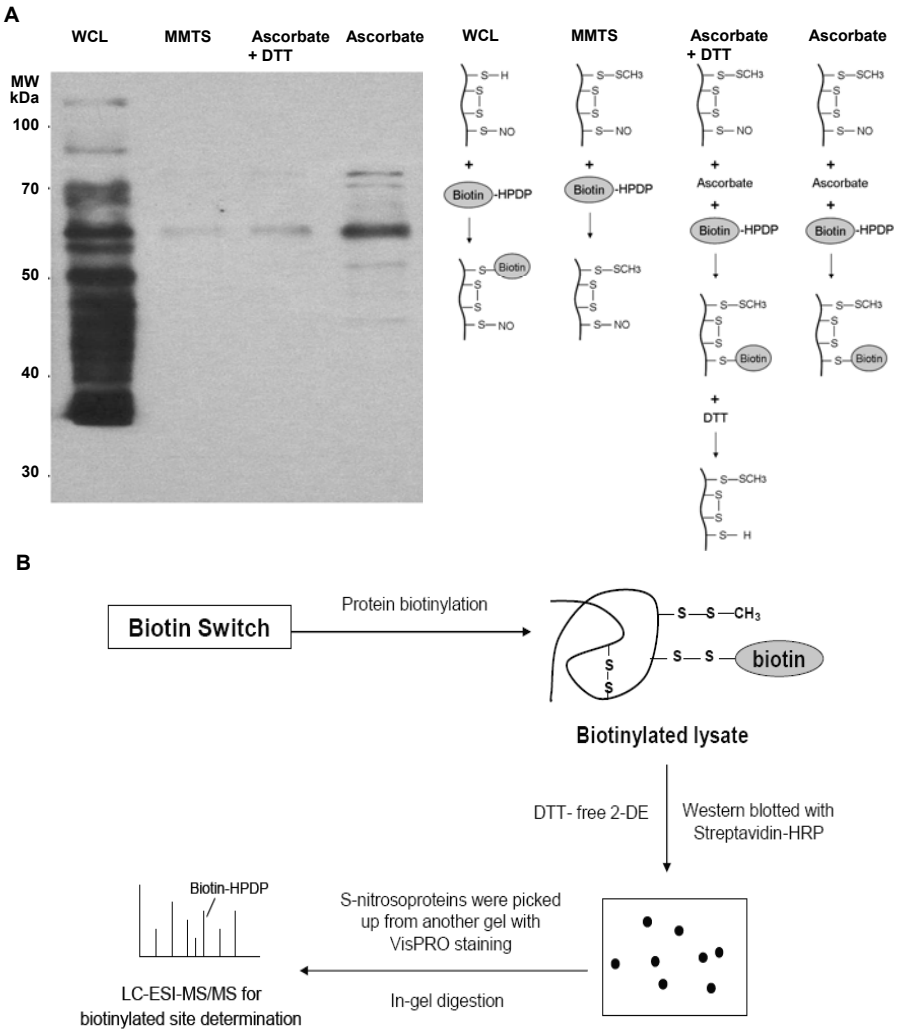


Fig. 1. Whole cell lysate (WCL), MMTS-blocked lysate (MMTS) and ascorbate-reduced lysate (Ascorbate) were collected at each step of the biotin-switch procedure. A negative control was prepared by adding DTT (Ascorbate + DTT). Each reactant was separated using SDS-PAGE and subjected to Western blotting with streptavidin-HRP prior to development on X-ray films. The binding of biotin-HPDP to cysteine residues under different conditions is illustrated (A). The lysate was subjected to the biotin-switch assay and then to Western blotting in order to analyze posttranslationally modified S-nitrosoproteins (B).

cysteine thiols were blocked with MMTS. A subsequent ascorbate treatment, an essential step to reduce S-NO bond, recovered the protein that could be biotin labelled. Moreover, these ascorbate-recovered biotin labelling proteins were almost blocked by 1% (w/v) DTT. The binding specificity of biotin-HPDP is illustrated (Fig. 1A). Then, the biotin switch-based 2-DE steps were used to analyze S-nitrosoproteins that did not involve DTT (Fig. 1B).

RESULTS

The effect of GT and screening of S-nitrosoproteins

To determine the GT effect on S-nitrosylation of proteins, the optimal effective treatment condition was depended on concentrations of crude extract of GT and treatment duration. Using the different concentrations (control, 1, 5, 10 and 20 μg/ml) and duration (0.5, 1 and 6 h), the most adequate treatment condition was concentration of 10 μg/ml and treatment duration of 6 h because the other conditions' relative density had all been below its peak level (Fig. 2). After the binding status of the biotinylated proteins was confirmed, DTT-free 2-DE was used to screen S-nitrosoproteins (Fig. 3A). A total of seven proteins with decreased S-nitrosylation and two proteins with enhanced S-nitrosylation were detected. Further, posttranslational modification was also confirmed by comparing the density of the protein spots on the gels and X-ray films (Fig. 3B). The S-nitrosoproteins were identified from

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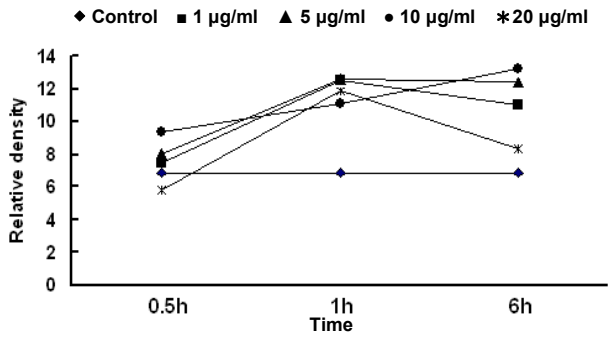


Fig. 2. Equal amounts of biotinylated lysate aliquots from series dilutions of GT extract and different treating time were separated by SDS-PAGE. The gel was Western blotted by streptavidin and developed using SuperSignal Femto regent. The relative density of each band was accumulated and statistically calculated using a densitometer.

another gel after GT treatment and subjected to mass spectrometric analysis (Fig. 3C). The seven proteins with decreased S-nitrosylation were included structural proteins such as vimentin (DS2, DS3 and DS5), tropomyosin 3, 4 (DS6 and DS7) and oxidative phosphorylation protein such as ATP synthase, F1 complex (DS1) and 80K-H protein (DS4). The two proteins with increased S-nitrosylation included the stress response protein, HspA9 (IS1), and the structural protein, beta-actin (IS2).

Determination of S-nitrosylated sites

The biotinylated site (Cys285) on the tryptic peptides of beta-actin could be determined using an nLC-ESI-MS/MS analyzer and the in-house MASCOT search program with a mass shift of 428.2 Da. (Figs. 4A & 4B). The relative ratios of the different modifications of the Cys285 residue of beta-actin, such as biotinylation (0.6%), methylthiolation (92.2%) and other changes (8.2%; acetylation, deamidation, Glu->pyro-Glu, etc.), were also estimated using MASIC label-free quantitation (Fig. 4C). The data confirmed that S-nitrosylation did occur in the Cys residues in endothelial proteins after GT treatment.

DISCUSSION

In the present study, several S-nitrosylation modified endothelial proteins treated by GT could be identified using this modified method. These changing nitrosoproteins belonged to a wide range of functional groups, such as stress-response protein (HspA9), cellular proteins involved in energy metabolism (ATP synthase-mitochondrial F1 complex), cell signalling protein (80K-H protein) and structural/contractile cytoskeletal proteins (vimentin, tropomyosin 3 and 4 and beta-actin).

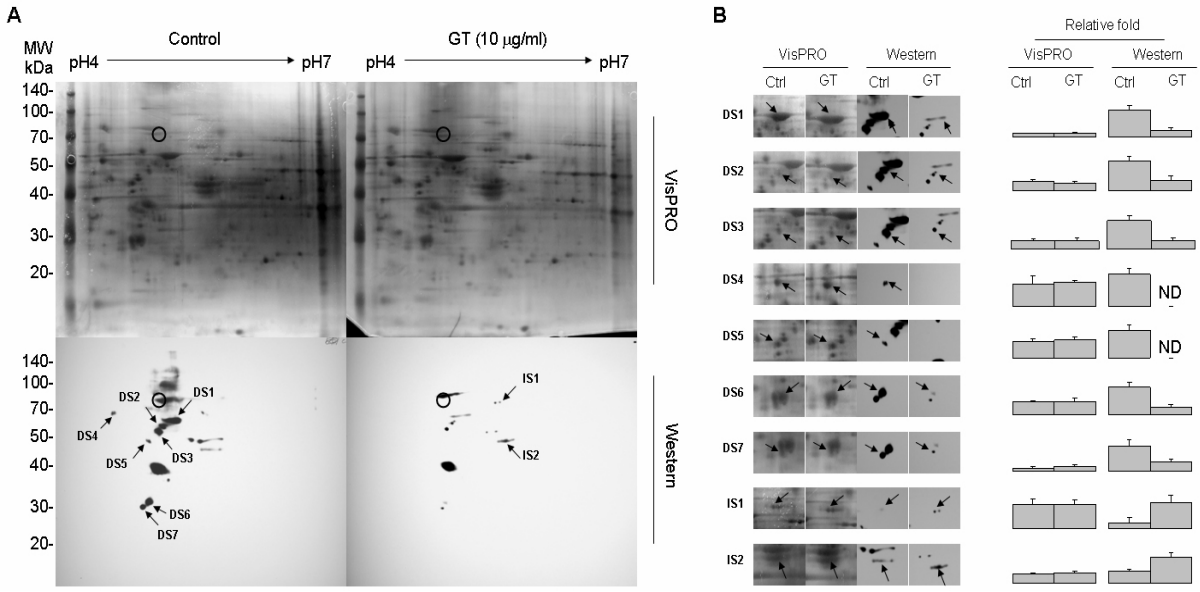
Previous studies reported that the anti-inflammatory property of GT can be attributed to its constituents, namely, triterpene, oleanolic acid, flavone, luteolin and luteolin-7-glucoside. It was demonstrated that the ethanol extract of GT inhibited the lipopolyssacharide (LPS)-induced NO, prostaglandin E2 (PGE2), IL-1β, IL-6, IL-12 and TNF-α synthesis in the murine macrophage cell line (Wu et al., 2005). It also exhibited a strong inhibitory effect on the release of TNF-α, IL-6 and IFN-γ in activated human whole blood and on the secretion of hepatitis B virus surface antigen (HBsAg) in the hepatocellular carcinoma cell line (Wu et al., 2005). The obtained results also demonstrate that the anti-inflammatory property of GT provides support for the traditional use of this plant in Chinese traditional medicine against inflammatory disorders (Wu et al., 2005).

NO is a ubiquitous regulator of cellular signalling. S-Nitrosylation inhibits and denitrosylation facilitates the sequential activation of caspases within macromolecular complexes (Matsumoto et al., 2003; Mannick et al., 1999). A well-characterized example of functional regulation by S-nitrosylation is the control of caspase-3-dependent death signalling. The mechanism of inhibition of apoptosis may involve the expression of protective genes such as Hsps and Bcl-2 as well as the direct inhibition of the pro-apoptotic caspase family proteases by S-nitrosylation of cysteine thiols (Choi et al., 2002).

S-nitrosylation of beta-actin was shown to inhibit the neutrophil integrin function (Thom et al., 2008). Vimentin is a member of the intermediate filament family of proteins associated with the cytoskeleton. Tropomyosin 3, 4 are skeletal muscle proteins. Tropomyosin 3 is normally found only in skeletal muscles and may substitute for α-tropomyosin in the specialized cells of the conduction system (Pieples et al., 2002). Tropomyosin 4 appears to regulate osteoclast motility and bone resorption by stabilizing actin filaments within podosomes and the sealing zone (McMichael et al., 2008). Whether the S-nitrosylation of tropomyosin 3, 4 would enhance their function remains to be determined. 80K-H biological function is poorly understood. Several studies propose a role in cell activation pathways through receptors located at the plasma membrane. 80K-H is also implicated in signalling and auto-regulation of the advanced glycosylation end products receptor, which participates in endocytic and cell-activation pathways, and is linked to nephropathy in type 1 diabetic patients (Gkika et al., 2004).

Among these proteins, increased posttranslational modification of HspA9 and decreased ATP synthase-mitochondrial F1 complex, respectively, were

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No.	Protein name	No.	Protein name
DS1	ATP synthase, F1 complex	DS6	Tropomyosin 3
DS2	Vimentin	DS7	Tropomyosin 4
DS3	Vimentin	IS1	HSPA9
DS4	80K-H protein	IS2	Beta actin
DS5	Vimentin		

Fig. 3. The gels were rapidly stained with VisPRO dye to premark the locations of all proteins and then destained with SDS-PAGE running buffer. The numbers denoted on the X-ray film after mock treatment represent the S-nitrosoproteins whose production was inhibited or enhanced (A). Evidence regarding the post- translational modification of the 9 S-nitrosoproteins is illustrated separately, and the spot densities were calculated using the results of 3 experiments. ND: Not detected (B). S-Nitrosoproteins in EAhy926 cells were detected using nLC-MS/MS (C).

A

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  1 MDDDIAALVV DNGSGMCKAG FAGDDAPRAV FPSIVGRPRH QGVMVGMGQK
 51 DSYVGDEAQS KRGILTLKYP IEHGIVTNWD DMEKIWHHTF YNELRVAPEE
101 HPVLLTEAPL NPKANREKMT QIMFETFNTP AMYVAIQAVL SLYASGRTTG
151 IVMDSGDGVT HTVPIYEGYA LPHAILRLDL AGRDLTDYLM KILTERGYSF
201 TTTAEREIVR DIKEKLCYVA LDFEQEMATA ASSSSLEKSY ELPDGQVITI
251 GNERFRCPEA LFQPSFLGME SCGIHETTFN SIMKCDVDIR KDLYANTVLS
301 GGTTMYPGIA DRMRKEITAL APSTMKIKII APPERKYSVW IGGSILASLS
351 TFQQMWISKQ EYDESGPSIV HRKCF
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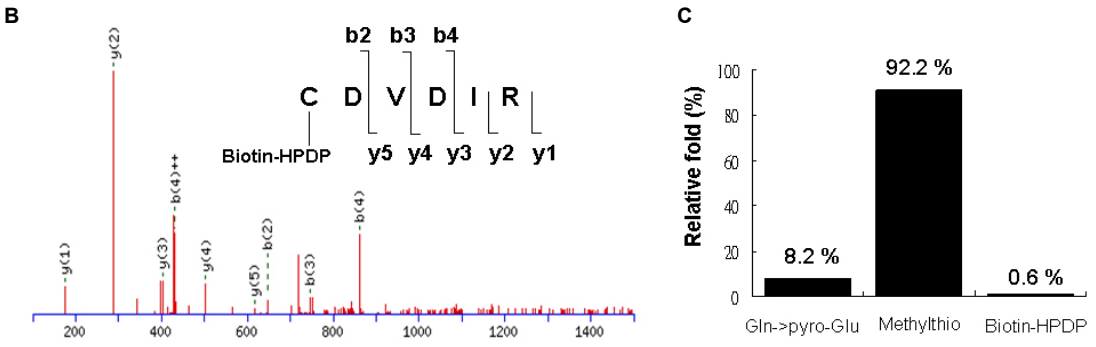


Fig. 4. MS/MS data for all peptide ions were searched against the SwissProt database using the in-house MASCOT algorithm. One of the biotinylated peptides was identified because of a mass shift of 428.2 Da. The biotin-HPDP bound peptide sequence of beta-actin is highlighted (A). The peptides detected using MS/MS analyses are shown (B). The relative ratio of the variable modifications of Cys285 in beta-actin was obtained on label-free quantitation using the MASIC software (C).

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the main findings of functional proteins that might involve ROS synthesis and antioxidant reaction. These effects might contribute to the protection against endothelial damage. S-nitrosylation of HspA9 by GT treatment was enhanced via posttranslational stimulation of enzymatic activity. HspA9 protects cells from damage due to stress and may reduce intracellular ROS levels. The induction of Hsp70 represents an extremely conserved response to many different cellular injuries, including ROS and cytokines in vitro or in animal models of acute respiratory distress syndrome (ARDS) (Durand et al., 2000). However, the effects of S-nitrosylation of Hsp70 on ECs are still unclear. Thus, S-nitrosylation prevents the overproduction of NO, which might be harmful to endothelial cells because it can induce shear stress (Martínez-Ruiz et al., 2005).

Decrease in the S-nitrosylation of ATP synthase due to GT treatment may interfere with ATP production and ROS formation. Extracellular nucleotides such as ATP play an important role in regulating the generation of ROS and thereby modulate inflammation, mediator production, cell-mediated killing and apoptosis. The fact that both dinucleotides (ADP) and trinucleotides (ATP) promote ROS formation indicates the involvement of one or more P2 receptors (Guerra et al., 2007). The activity of ATP synthase (ATP production) depends on the cellular capacity for oxidative phosphorylation; this activity determines the susceptibility of a cell to ROS-dependent cell death mediated through a mitochondria-dependent pathway (Santamaría et al., 2006). No matter increasing S-nitrosylation of HspA9 or decreasing S-nitrosylation of ATP synthase, F1 complex, these effects all contribute to reduce ROS formation and partially explained the GT anti-oxidant effect on ECs.

Currently, the scarcity of S-nitrosoproteins in vivo is the most difficult challenge in studying the physiological functions of posttranslational S-nitrosylation of proteins. The biotin-switch method is a more superior procedure than the other currently used methods (Huang et al., 2009). We modified this method and were able to perform our experiment with only two petri dishes (10 cm) containing cultured ECs. Two key points should be noted here. One is that streptavidin is highly sensitive and can detect very low amounts of proteins up to 1-10 pg. Therefore, the biotinylated lysate could be subjected to 2-DE, and streptavidin-HRP was used to screen S-nitrosoproteins that were present in the lysate. The other key point is the rapid staining of the gel with VisPRO fast staining dye. Since fixation with acetic acid was not required, the yield of tryptic peptides from the gel slices was higher, which in turn increased the accuracy of the mass spectrometric analysis. The GT extract components may mediate the posttranslational S-nitrosylation of functional proteins. This is a novel mechanism via which GT interferes with the antioxidant functions of ECs. S-nitrosylated proteins can be used in further studies on the importance of GT in cardiovascular protection and in studies on signalling pathways involving different antioxidants and anti-inflammatory reactions. However, more specific downstream effects of these S-nitrosoproteins need to be investigated further.

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香茹的粗萃取物對血管內皮細胞的蛋白質體及轉譯後硝酸化蛋白質體之研究

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摘要：香茹草（*Glossogyne tenuifolia*）是一種天然傳統藥草且已經被報導藉由抑制細胞中自由基活性氧分子（free reactive oxygen species, ROS）來達到抗氧化及保護血管內皮細胞之作用。本研究之目的為藉由蛋白質體學來探討香茹草保護內皮細胞之機轉。藉由modified biotin-switch之實驗方法來分析蛋白質之硝酸化情形，以此方法來偵測香茹草在蛋白質經轉譯後硝酸化上所帶來的反應與作用。實驗結果發現，經香茹草萃取物作用後的血管內皮細胞，其中HspA9 (IS1)與beta-actin (IS2)此二種轉譯後硝酸化蛋白質有提昇的現象；vimentin (DS2、DS3 及 DS5)、tropomyosin 3, 4 (DS6 及 DS7)與oxidative phosphorylation protein（如ATP synthase, F1 complex (DS1)與80K-H protein (DS4)）等七種轉譯後硝酸化蛋白質則受到抑制。由於HspA9經轉譯後硝酸化可能減少血管內皮細胞ROS，且ATP synthase經轉譯後硝酸化可能干擾ATP的產生及ROS的形成，因此本研究結果指出香茹草具有保護內皮細胞損傷之另一新的機轉，可能是藉由抑制細胞中氧化物質的形成所引發。

關鍵詞：抗氧化、自由基活性氧分子、香茹草、轉譯後硝酸化、血管內皮細胞。

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