A Proteomic Approach for Evaluating the Effects of Proanthocyanidins on Rotenone-Induced Toxicity in SH-SY5Y Neuroblastoma Cells

Cristobal L. Miranda, Woon-Gye Chung, Hui-Jing Li, Jan F. Stevens and Claudia S. Maier
Oregon State University, Corvallis, Oregon

OVERVIEW

Purpose. Determine the potential protective effects of proanthocyanidins against cellular injury caused by rotenone in a human neuroblastoma cell line, SH-SY5Y cells, with emphasis on apoptosis and protein carbonylation.

Methods. Cytotoxicity of rotenone in SH-SH5Y cells, with or without pretreatment with proanthocyanidins (PCs), was evaluated by the LDH assay. Apoptosis was measured using caspase-3 activity and by Western blots using anti-PARP antibodies. Protein carbonylation was determined using a carbonyl-specific labeling reagent and NeutrAvidin staining; MALDI-MS/MS was used to identify carbonylated proteins.

Results. PC monomers and dimers protected SH-SY5Y cells against rotenone toxicity whereas the PC mixture, at high concentrations, enhanced the toxicity of rotenone in SH-SY5Y cells. However, none of the PCs was effective in protecting against rotenone-induced apoptosis in SH-SY5Y cells. Carbonylated proteins in SH-SY5Y cells identified by MALDI tandem mass spectrometry were α-enolase, cytoplasmic 1 β-actin, glyceraldehyde 3-phosphate dehydrogenase, peptidyl-propyl cis-trans isomerase A, histone H4, and ubiquitin.

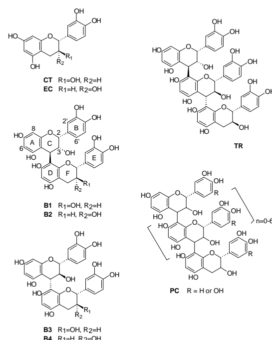
INTRODUCTION

The proanthocyanidins (PCs) are oligomers of polyhydroxy flavan-3-ol units such as catechin, epicatechin, and their epimers. As antioxidants, PCs may have potential use in the prevention of certain cardiovascular and neurodegenerative diseases. Hops (*Humulus lupulus*) contain oligomeric PCs consisting of catechin, (-)-epicatechin and epigallocatechin subunits ranging from monomers to polymers.

Rotenone, a complex 1 inhibitor, is known to induce apoptosis in the neuroblastoma cell line, SH-SY5Y, through enhanced mitochondrial reactive oxygen species (ROS) production. Rotenone-induced dopaminergic neuronal injury has been used as a model of Parkinson's disease. Since rotenone toxicity may be mediated by ROS, we hypothesized that PCs which are potent antioxidants may provide protection against rotenone-induced toxicity in SH-SY5Y cells. This study evaluates the protective effects of PC monomers, dimers, oligomers and PC mixture isolated from hops against the cytotoxicity of rotenone in SH-SY5Y cells, with emphasis on apoptosis and on protein carbonylation. Human diseases associated with protein carbonylation include Parkinson's disease and Alzheimer's disease. Carbonyl derivatives are formed by a direct metal catalyzed oxidative attack on the amino-acid side chains of proline, arginine, lysine, and threonine or by secondary reactions with reactive carbonyl compounds on carbohydrates (glycoxidation products), lipids, and advanced glycation or lipoxidation end products.

MATERIALS AND METHODS

Test compounds. Proanthocyanidin (PC) monomers (catechin and epicatechin, dimers (B1–B4), trimer [epicatechin-(4β→8)-catechin-(4α→8)-catechin], tetramers, pentamers, and mixtures of oligomers were extracted from hops (*Humulus lupulus* L. cv. Willamette). The structures of PCs are shown in below.



Chemical structures of catechin (CT), epicatechin (EC). dimers (B1 to B4), trimer [TR, epicatechin-(4β→8)-catechin-(4α→8)-catechin], and oligomeric PCs.

Cell culture studies. The protective effect of the test compounds on rotenone-mediated toxicity in SH-SY5Y cells was determined by LDH released into the culture medium. SH-SY5Y cells were plated in 6-well or 96-well plates were pretreated with PCs for 30 min before adding rotenone (10 μM) to the culture medium. Twenty-four hr after rotenone exposure, the media were collected for LDH assay and the cells attached to the plaltes were harvested by using a cell scraper. The cells were disrupted by sonication, centrifuged at 10,000 g and the resulting supernatant was used for SDS-PAGE and Western blotting.

Apoptosis Studies. Western blotting was used to measure the release of cytochrome c into the cytosol and to determine cleavage of caspase-3 and of PARP in 10K supernatants of SH-SY5Y cells. The blots were probed with antibodies to cytochrome c, pro (full-length) and active/cleaved caspase-3 (Imgenex Corporation, San Diego, CA) or with rabbit anti-human PARP (Roche Molecular Biochemicals, Germany).

Western blot analysis of SH-SY5Y cell proteins after reaction with ARP. We have conducted a preliminary study to determine the presence of oxidatively modified proteins in SH-SY5Y Cells using the probe, N'-aminooxymethylcarbonylhydrazino D-biotin (or aldehyde-reactive probe, ARP). In this assay, proteins with aldehyde/keto functionalities will react with ARP and the ARP-labeled (biotinylated) proteins are then detected by affinity staining with Avidin-Horseradish Peroxidase Conjugate (NeutrAvidin, from Molecular Probes) in Western blots.

SDS-PAGE analysis of SH-SY5Y cell proteins after reaction with ARP. To identify the proteins recognized by ARP in the Western blot analysis, protein samples (50 μg) from control and treated SH-SY5Y cells were reacted with 5 mM ARP and then subjected to SDS-PAGE. The bands of interest were excised, digested with trypsin and the resulting peptides were analyzed with MALDI tandem mass spectrometry.

RESULTS

Figure 1. All the individual proanthocyanidin fractions, from monomers to trimer, are effective in protecting SH-SY5Y cells from the toxic effects of rotenone (5 μM). However, the PC mixture (10 μg/ml) potentiated the toxicity of rotenone.

Figure 2. PC alone (5 μg/ml) or rotenone alone (10 μM) produced a significant increase in caspase-3 activity of SH-SY5Y cells. When SH-SY5Y cells were treated with 5 μg/ml PC prior to rotenone exposure, caspase-3 activity of cell lysates was enhanced, indicating a synergistic effect of PC on rotenone-induced activation of caspase-3 in these cells. Increasing the PC concentration to 25 μg/ml resulted in a marked decrease in rotenone-induced activation of caspase-3 in these cells which could be due to increased cell death by necrosis.

Figure 3. Cells treated with 10 μM rotenone (lane 2) and 25 μg/ml PC mixture (lane 4) showed cleavage of PARP to an 89-kDa fragment. However, the 89 kDa fragment was of less intensity in cells treated first with 25 μg/ml PC mixture and then with 10 μM rotenone (lane 6), suggesting a reduction in caspase activation.

Figure 4. Proteins that reacted with ARP as seen in Western blots were identified by MALDI-MS/MS as α-enolase, cytoplasmic 1 β-actin, glyceraldehyde 3-phosphate dehydrogenase, peptidyl-propyl cis-trans isomerase A, histone H4, and ubiquitin.

Figure 5 and Table 1. MALDI-TOF MS spectrum of the tryptic in-gel digest of a protein band excised from a SDS-PAGE gel; MS/MS of the 5 annotated precursor ions (Figure 5) yielded sequence information (Table 1) that led to the identification of this protein as α-enolase.

Figure 6 and Table 2. MALDI-TOF MS spectrum of a tryptic in-gel digest of excised protein band; MS/MS of the 8 annotated precursor ions (Figure 6) yielded fragment ion spectra that led to the identification of glyceraldehyde-3-phosphate dehydrogenase (GADPH). A peptide fragment (*m/z* 1795) of GADPH at position 309 – 322 shows tryptophan oxidation by MS/MS analysis (Table 2).

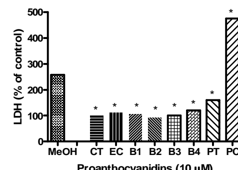


Figure 1. LDH activity in media of SH-SY5Y cells exposed to rotenone (5 μM) in the presence and absence PC fractions. Asterisk (*) indicates a significant difference from control (methanol) values, $p < 0.05$. $N = 4$.

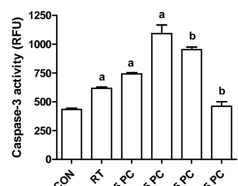


Figure 2. Effect of PC mixture, alone or in combination with rotenone (RT, 10 μM), on caspase-3 activity in SH-SY5Y cells. Cells were treated with the compounds for 24 h and the catalytic activity of the cell lysates was assayed using the substrate, Ac-DEVD-AMC. Results are means ± SE ($n = 4$). A, $P < 0.05$ compared with control without rotenone treatment. b, $P < 0.05$ versus group treated with rotenone (RT) only.

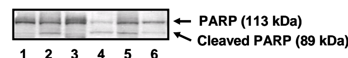


Figure 3. Effect of rotenone and PC mixture, alone or in combination, on poly(ADP-ribose polymerase (PARP) cleavage in SH-SY5Y cells. PARP cleavage was determined by Western blotting after the cells were exposed to the compounds for 24 h. Lane 1, control (without rotenone or PC mixture); lane 2, 10 μM rotenone; lane 3, 5 μg/ml PC mixture; lane 4, 25 μg/ml PC mixture; lanes 5 and 6, pretreatment with 5 and 25 PC mixture 30 min prior to 10 μM rotenone.

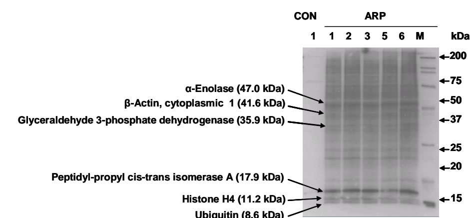


Figure 4. Western blot of SH-SY5Y cellular protein fractions after reaction with ARP, with detection of biotinylated proteins by affinity staining with NeutrAvidin. CON 1: DMSO-treated not labeled with ARP. ARP (samples reacted with ARP): 1 (DMSO), 2 (10 μM rotenone), 3 (5 μg/ml PC mixture), 5 (10 μM rotenone + 5 μg/ml PC mixture), 6 (10 μM rotenone + 25 μg/ml PC mixture).

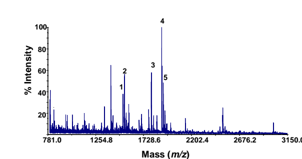


Figure 5. MALDI-TOF MS spectrum corresponding to α-enolase tryptic in-gel digest.

Table 1. Peptides identified by MALDI-MS/MS from a protein band in SDS-PAGE of a lysate of SH-SY5Y cells. The MS/MS data obtained from the tryptic peptide ions (Figure 5) identified the protein band as α-enolase (47 kDa).

Position	Observed mass	Calculated mass	Error (Da)	Peptide sequence	Ions score	Peak No.
32 - 49	1804.92	1803.94	-0.03	AAVPSGASTGIYEALELR	50	3
162 - 178	1907.96	1906.98	-0.03	LAMQEFMILPVGAANFR	48	4
162 - 178	1923.95	1922.97	-0.03	LAMQEFMILPVGAANFR oxidation (M)	17	5
239 - 252	1540.76	1539.78	-0.02	VVIGMDVAASEFFR	36	2
358 - 371	1525.75	1524.76	-0.02	LAQANGWGVMVSHR	49	1

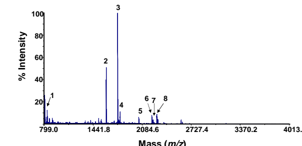


Figure 6. MALDI-TOF MS spectrum of the in gel-digest that yielded glyceraldehyde-3-phosphate dehydrogenase.

Table 2. Peptides identified by MALDI-MS/MS from a protein band in SDS-PAGE of a lysate of SH-SY5Y cells. The fragment ion spectra obtained from the marked precursor ions in Figure 6 identified the protein band as glyceraldehyde-3-phosphate dehydrogenase (35.9 kDa).

Position	Observed mass	Calculated mass	Error (Da)	Peptide sequence	Ions score	Peak no.
5 - 12	805.42	804.42	-0.007	VGVNGFGR	25	1
66 - 79	1613.89	1612.89	-0.013	LVINGNPITIFQER	60	2
66 - 83	2041.09	2040.10	-0.015	LVINGNPITIFQERDPSK	28	5
86 - 106	2277.02	2276.03	-0.013	WGDAGAEYVVESTGVFTTMEK	50	8
118 - 138	2213.09	2212.10	-0.019	VIISAPSADAPMFVMGVNHEK	91	6
118 - 138	2229.09	2228.10	-0.016	VIISAPSADAPMFVMGVNHEK oxidation	49	7
309 - 322	1763.79	1762.79	-0.013	LISWYDNEFGYSNR	97	3
309 - 322	1795.78	1794.78	-0.015	LISWYDNEFGYSNR Formylkynurenine (W)	25	4

CONCLUSIONS

- PC monomers, dimers and trimer protected SH-SY5Y cells against rotenone toxicity whereas the PC mixture, at 10 μg/ml, enhanced the toxicity of rotenone in SH-SY5Y cells.
- None of the PCs was effective in protecting against rotenone-induced apoptosis in SH-SY5Y cells.
- Carbonylated proteins in SH-SY5Y cells identified by MALDI tandem mass spectrometry: α-enolase (47 kDa), cytoplasmic 1 β-actin 41.6 kDa), glyceraldehyde 3-phosphate dehydrogenase (35.9 kDa), peptidyl-propyl cis-trans isomerase A (17.9 kDa), histone H4 (11.2 kDa), and ubiquitin (8.6 kDa).

ACKNOWLEDGEMENTS

This study was supported by the Agricultural Research Foundation of Oregon and by the National Institute on Aging grant no. AG025372.