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ORIGINAL ARTICLE

Proteomics analysis of human umbilical vein endothelial cells treated with resveratrol

Bin Shao · Mei Tang · Ziqiang Li · Rui Zhou · Yaqi Deng · Chunlai Nie · Zhu Yuan · Liangxue Zhou · Minghai Tang · Aiping Tong · Yuquan Wei

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Abstract In the past decade, the small polyphenol resveratrol has received widespread attention as either a potential therapy or as a preventive agent for numerous age-related chronic diseases, including cardiovascular atherosclerosis, cancer, hypertension, and diabetes, but the biological processes and molecular pathways by which resveratrol induces these beneficial effects, as well as its safety and toxicology remain largely undefined. To explore the molecular mechanisms of resveratrol involved in the amelioration of endothelial dysfunction and vascular disease, in the present study the protein profile changes of human umbilical vein endothelial cells in response to resveratrol treatment were investigated using proteomics approaches (2-DE combined with MS/MS). As a result, four down-regulated protein species named elongation factor 2 (EEF2), carboxymethyl-cofilin-1 (cofilin-1), acetyl-eukaryotic translation initiation factor 5A-1 (acetyl-EIF5A) and barrier-to-autointegration factor, and five up-regulated protein species named heat shock protein beta-1 (HSP27), phospho-HSP27, phospho-stathmin, Nicotinate-nucleotide pyrophosphorylase and 1,2-dihydroxy-3-keto-5-methylthiopentene dioxygenase were identified. Among them, two translation-related protein species (EEF2 and acetyl-EIF5A) were the most significantly changed (over tenfold). Phospho-EEF2 was further verified to be dramatically up-regulated by immunoblot assays. It is notable that in the present study several protein species with post-transcriptional modification (carboxymethyl-, acetyl-, and phospho-) were found to be altered following exposure to resveratrol. These findings may improve our understanding of the molecular mechanisms underlying the pleiotropic effects of resveratrol on endothelial cells.

Keywords Resveratrol · HUVECs · 2-DE · Proteomics

Introduction

With the rapid growth in the number of older population, there is a remarkable increase in the prevalence of age-related disorders including cardiovascular and neurodegenerative diseases, diabetes, hypertension, and cancer. The World Health Organization (WHO) estimated that 17 million people die annually from cardiovascular disease—more than from any other cause. Cardiovascular disease accounts for one in three of all deaths in the United States and the incidence will continue to rise (Aging Statistics 2010; Roger et al. 2011). So, the search for interventions that can prevent and/or alleviate age-related health problems is attracting more and more attention.

Resveratrol (Resv) is a naturally occurring polyphenol found in more than 70 species of plants, including peanuts, mulberries, and grapes. In the past decade, Resv has received widespread attention as either a potential therapy or as a preventive agent for numerous age-related chronic diseases, including cardiovascular atherosclerosis, cancer, hypertension, and diabetes (Shishodia and Aggarwal 2006; Harikumar and Aggarwal 2008). A number of long-term

Bin Shao and Mei Tang contributed equally to this work.

B. Shao · M. Tang · Z. Li · R. Zhou · Y. Deng · C. Nie · Z. Yuan · M. Tang · A. Tong (✉) · Y. Wei
State Key Laboratory of Biotherapy and Cancer Center, West China Medical School, West China Hospital, Sichuan University, Chengdu, People's Republic of China
e-mail: aipingtong@gmail.com

L. Zhou
Department of Neurosurgery, West China Hospital, Sichuan University, Chengdu, People's Republic of China

clinical studies in humans have recently been initiated or are under planning (Smoliga et al. 2011).

In France, an important dietary source of Resv is red wine, and wine consumption is often postulated to be an important factor in the French paradox, a term used to describe the observation that the French population has a comparatively low incidence of cardiovascular disease, despite a diet high in saturated fats (Kopp 1998). Nutritional supplements of Resv can be produced by extraction of Resv from the roots of *Polygonum cuspidatum* (knotweed), which has been used in traditional Asian medicine to treat a range of diseases including cardiovascular disorders. Resv has been shown to confer cardioprotective effects in animal models of diabetes mellitus, improving endothelial function and attenuating vascular inflammation. Similar protective effects of Resv treatment were observed in aged mice. Moreover, the consumption of Mediterranean-style diets, which are rich in Resv, is associated with a reduced risk of cardiovascular mortality in humans (Bertoni et al. 2006; Pearson et al. 2008; Ungvari et al. 2007; Dolinsky and Dyck 2011).

In spite of the potentially beneficial effects on some age-related diseases, the molecular targets through which Resv acts have not yet been fully determined. Although there is evidence that some of Resv's actions are mediated by activation of the SIRT1 deacetylase, the hypothesis that Resv as a SIRT1 activator has been questioned in recent studies. In addition, Resv has been reported to inhibit the insulin signaling pathway independently of SIRT1, suggesting that alternative mechanism(s) for the Resv effects may exist. Indeed Resv has been shown to mediate some of its effects through inhibition of cyclo-oxygenases (Baur et al. 2006; Pacholec et al. 2010; Lagouge et al. 2006; Beher et al. 2009; Wood et al. 2004; Barger et al. 2008; Zhang 2006) and could therefore act through some of the same mechanisms as aspirin. The eukaryotic translation initiation machinery was also reported to be a molecular target for Resv (Lomenick et al. 2009). A study to select Resv-binding proteins revealed quinone reductase 2 as one of the highest-affinity targets, although the significance of this observation is not yet known (Buryanovskyy et al. 2004). When used at a high concentrations, Resv was shown to have cancer chemopreventive activity (Jang et al. 1997; Tseng et al. 2004). Recent studies showed that Resv can attenuate mitochondrial oxidative stress and induce mitochondrial biogenesis in endothelial cells (Ungvari et al. 2009; Csiszar et al. 2009). Collectively, the complex mechanisms underlying the numerous beneficial effects of Resv remain to be fully elucidated.

Vascular endothelial dysfunction plays an important role in the pathogenesis of various vascular diseases. In the present study, 2DE–MS strategy was utilized to analyze the alterations in protein profile of HUVECs following Resv treatment with the aim to improve the understanding of the molecular mechanisms underlying the pleiotropic effects of Resv on endothelial cells.

Materials and methods

Cell culture and treatment

HUVECs were purchased from ATCC (Rockville, MD, USA). Resv was purchased from sigma (St. Louis, MO, USA) and was dissolved in dimethyl sulfoxide (DMSO) as 100 mM stock solution. Cells were maintained in DMEM (Gibco,USA) containing 10% fetal calf serum (Hyclone, USA), penicillin (10^7 U/L), and streptomycin (10 mg/L) at 37°C in an atmosphere containing 5% CO_2. For 2-DE analysis, HUVECs were treated with 100 μM Resv for 48 h. Control cells were cultured in a medium containing equal amount of DMSO instead of Resv solution. The cells were collected by centrifugation and washed twice with PBS, and were subsequently transferred to sterile plastic tubes for storage at −80°C until use.

Flow cytometry assays

Flow cytometry analysis using propidium iodide (PI) staining was performed to measure apoptosis. After incubation with resveratral (0, 5, 20, 50, 100, 150, and 200 μM) for 48 h, HUVECs were washed with PBS twice and harvested by trypsinization. The cells were washed again with PBS and fixed by incubation in 50% ice-cold ethanol/PBS for 30 min on ice. After washing with PBS once more, the cells were re-suspended and incubated in PI solution (70 μM propidium iodide, 38 mM sodium citrate and 20 μg/ml RNase A) for 30 min at 37°C. Flow cytometry analyses were performed on a FACScan flow cytometry system (Becton-Dickinson, San Jose, CA). Three independent experiments were carried out.

2-DE and image analysis

2-DE was performed as described previously with minor modifications (Tong et al. 2008a, b). Briefly, cells were homogenized and sonicated in lysis buffer (7 M urea, 2 M thiourea, 100 mM DTT, 4% CHAPS, 0.2% pH 3–10 ampholyte, Bio-Rad, USA) containing a protease inhibitor cocktail. Samples (2 mg in 300 μl) were applied to IPG strips (17 cm, pH 3–10 NL, Bio-Rad) by passive rehydration for 12–16 h. After IEF and equilibration in SDS, the second dimension was performed on a 12% SDS-PAGE gel. The gels were stained with CBB R-250 (Merck, Germany) and scanned with a Bio-Rad GS-800 scanner. 2-DE analyses were independently repeated three times. Gel

maps were analyzed with the PDQuest software Version 6.1 (Bio-Rad). The quantity of each spot in a gel was normalized as a percentage of the total quantity of all spots in that gel and evaluated in terms of OD. Student's t test was performed to compare data from the three experiments. Only those spots that changed consistently and significantly (more than twofold, $p < 0.05$) were selected for analysis with MS.

In-gel digestion

In-gel digestion of protein species was carried out using MS-grade trypsin (Promega, Madison, WI, USA) according to the manufacturer's instructions. In brief, spots were cut out of the gel (as plugs 1–2 mm in diameter) using a razor blade. The spots were destained twice for 45 min at 37°C with 100 mM NH_4HCO_3/50% acetonitrile. Afterward, the gels were preincubated in 10–20 μL trypsin solution for 1 h. Then, at least 15 μL digestion buffer (10% acetonitrile/40 mM NH_4HCO_3) was added to cover the gels before an overnight incubation at 37°C. Tryptic digests were extracted using MilliQ water at first, followed by two extractions, 1 h each, with 50% acetonitrile/5% trifluoroacetic acid. The combined peptides were collected and dried in a vacuum concentrator at room temperature. Then, the samples were subjected to MS analysis.

MS/MS analysis and protein identification

ESI-Q-TOF MS/MS analysis and protein identification were performed as described previously with minor modifications (Tong et al. 2008a, b). Briefly, mass spectra were acquired using a Q-TOF mass spectrometer (Micromass, Manchester, UK) coupled with an ESI ion source (Micromass). For MASCOT analysis, peptide and fragment mass tolerance were set at 0.1 and 0.2 Da, respectively. Only protein species with probability-based MOWSE scores that exceeded the threshold ($p < 0.05$), and with their MW and pI consistent with the gel regions from which the spots were excised, were considered to be positively identified.

Immunoblot

Cells were homogenized and sonicated in RIPA buffer (50 mM Tris-base, 150 mM NaCl, 1.0 mM EDTA, 0.1% SDS, 1% Triton X-100, and 1% sodium deoxycholate) containing a protease inhibitor cocktail. Sample concentration was evaluated with the DC protein assay kit (Bio-Rad). Sample proteins were separated by 12% SDS-PAGE and transferred to PVDF membranes. Membranes were blocked overnight with TBS containing 0.1% Tween 20 in 5% skimmed milk at 4°C and then incubated with primary antibodies for 2 h at 37°C (anti-EEF2, diluted 1:10,000, ab75748, Abcam UK; anti-EEF2 (phospho-T56), diluted 1:300, ab53114, Abcam). After three washes in TBST, the membranes were incubated with a HRP-conjugated secondary antibody for 2 h at room temperature, and the positive bands were then visualized using enhanced chemiluminescence reagents (Millipore).

Results

Flow cytometry analysis

To assess the cytotoxicity of Resv on the HUVECs and determine the appropriate concentration for testing, flow cytometry assays were performed as described under "Methods." As shown in Fig. 1, after treatment with Resv at a concentration over 150 μM for 48 h a significant increase was observed in the percentage of apoptotic cells (Sub-G1). These data indicate that Resv exerts a significant cytotoxic effect on HUVECs when used at higher concentrations. Accordingly, the 2-DE analysis in this study was performed on cells treated with Resv at 100 μM concentration.

2-DE analysis

The alteration of protein species in HUVECs following treatment with Resv were assessed by 2-DE analysis in comparison with DMSO as control. A pair of representative 2-DE maps is shown in Fig. 2. While most of the spots did not change significantly, nine spots displayed marked and statistically significant alterations (± over twofold, $p < 0.05$) in HUVECs treated with Resv in comparison with control cells. The two spots (#1 and #3) associated

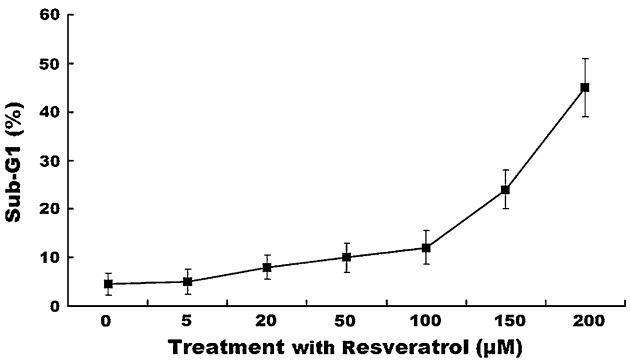

Fig. 1 Flow cytometry analysis of the effects of resveratrol on HUVECs. After treatment for 48 h with the indicated concentrations of resveratrol, the cells were stained with PI and analyzed by flow cytometry. There was a significant increase in the percentage of apoptotic cells (Sub-G1) after treatment with resveratrol at a concentration above 150 μM

with the most remarkable changes are shown in enlarged form in Fig. 3.

MS/MS Identification

All of the nine spots were positively identified by ESI-Q-TOF MS/MS analysis (Table 1). The average values of MOWSE score, number of unique peptides, and sequence coverage of the nine identified protein species were, respectively, 240, 8, and 46%. It is notable that several protein species found to be significantly altered following exposure to Resv had post-transcriptional modifications (carboxymethyl-CFL1, acetyl-EIF5A, phospho-HSP27 and phospho-STMN1) (Table 1). A representative MS spectrum of spot #3 is shown in Fig. 4.

Immunoblot validation

As shown in Fig. 5, consistent with the observations in 2-DE analysis, EEF2 was down-regulated in HUVECs after exposure for 48 h to various concentrations of Resv (1, 5, 10, 20, 50, and 100 μM) in comparison with DMSO-treated control cells. Resv could down-regulate the level of total EEF2 even at lower concentrations (1 μM). Conversely, Resv could up-regulate phospho-EEF2 (T56) only at higher concentrations (100 μM).

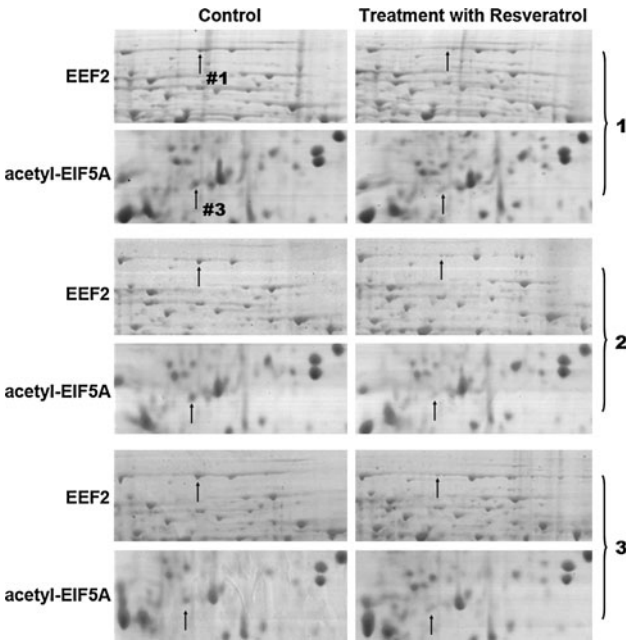

Fig. 3 Enlargement of selected regions. *Spots* # 1 (EEF2) and # 3 (Acetyl-EIF5A) were consistently and significantly down-regulated in HUVECs after treatment with resveratrol in three independent experiments

Discussion

In the present study, HUVECs were used as a model, and a 2DE-based proteomic approach was undertaken to annotate the protein species whose levels are altered in HUVECs after treatment with Resv. The proteomic analysis detected a total of nine altered protein species, whose functions are connected with diverse biological processes such as NAD biosynthesis, transcription, signal transduction, DNA binding, and molecular chaperoning. Among them, four protein species were down-regulated and five protein species were up-regulated; in detail, several protein species with post-transcriptional modifications (carboxymethyl-, acetyl-, and phospho-) were found to be altered following exposure to Resv.

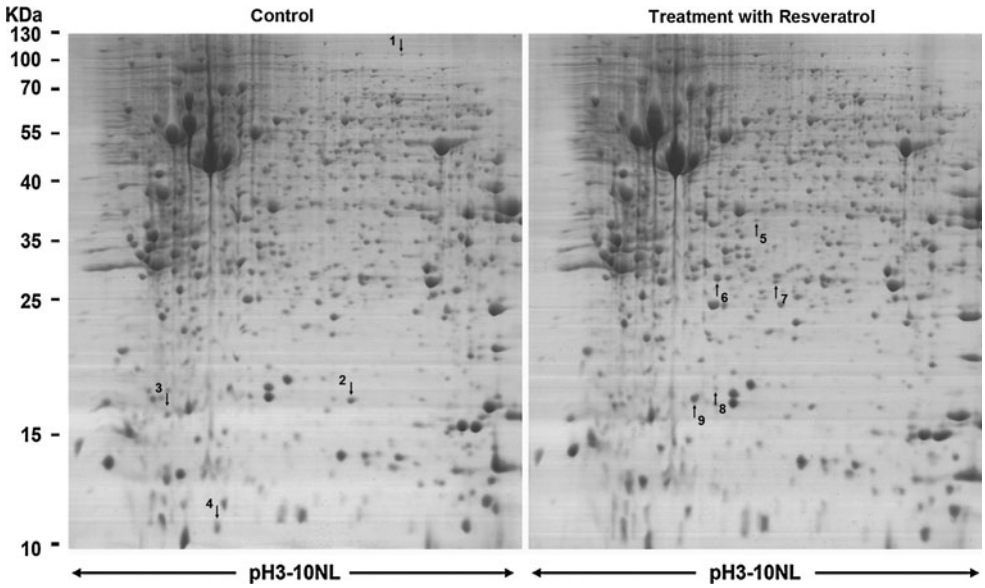

Fig. 2 Representative 2-DE gel images of HUVECs. Total protein extracts from DMSO-treated control cells (*left panel*) and resveratrol-treated cells (*right panel*) were separated on pH 3–10 nonlinear IPG strips in the first dimension followed by 12% SDS-PAGE in the second dimension. The gels were visualized by CBB staining. In total, nine changed spots were identified by 2-DE and MS/MS analysis (marked with *arrow* and *number*). Information on each of these protein species is reported in Table 1

Table 1 Identified protein species by MS/MS analysis

Spot no.	Protein description	Gene name	Function	Accession no.	Theoretical Mr/pI[a]	Score[b]	No.of pep. and cove.[c] (%)	Fold change[d]
1	Elongation factor 2	EEF2	Transcription	P13639	96,246/6.41	136	13/26	↓ >10
2	Cofilin-1	CFL1	Signal transduction	P23528	18,719/8.22	400	12/62	↓ 2.1 ± 0.7
3	Eukaryotic translation initiation factor 5A-1	EIF5A	Transcription	P63241	17,049/5.08	406	8/81	↓ >10
4	Barrier-to-autointegration factor	BANF1	DNA binding	O75531	10,280/5.81	84	3/42	↓ 4.3 ± 1.1
5	Nicotinate-nucleotide pyrophosphorylase (carboxylating)	QPRT	NAD biosynthesis	Q15274	31,138/5.81	156	4/17	↑ 3.8 ± 1.2
6	Heat shock protein beta-1	HSPB1	Chaperone	Q9UC36	22,826/5.98	298	8/49	↑ 2.0 ± 0.5
7	Heat shock protein beta-1	HSPB1	Chaperone	Q9UC36	22,826/5.98	306	10/56	↑ 2.3 ± 0.7
8	1,2-Dihydroxy-3-keto-5-methylthiopentene dioxygenase	ADI1	Metabolism	Q96P85	21,542/5.43	101	6/39	↑ 2.5 ± 0.8
9	Stathmin	STMN1	Signal transduction	P16949	17,292/5.76	272	8/41	↑ 2.7 ± 0.9

[a] Theoretical molecular weight (kDa) and p*I* from the ExPASy database

[b] Probability-based MOWSE scores

[c] The number of unique peptides identified by MS/MS sequencing (multiple matches to peptides with the same primary sequence count as one)

[d] Average fold changes before and after resveratrol treatment from three independent analyses (↑, increase; ↓, decrease)

EEF2 occupies an essential role in protein synthesis as it catalyzes the translocation on the 80S ribosome of mRNA and of the two tRNAs after peptidyl transfer. EEF2 is the sole substrate of EEF2 kinase (EEF2 K), and phosphorylation of EEF2 by EEF2 K is known to inhibit EEF2-ribosome binding and thereby to arrest mRNA translation (Jørgensen et al. 2006; Ryazanov et al. 1988). Numerous studies have revealed that protein synthesis is a determinant of aging in diverse organisms such as yeast, worms, flies, and mice and can thus be considered as a universal component of the aging process (Tavernarakis 2008). Recent studies have shown that Resv could inhibit changes in association between EIF4E and EIF4G, and phosphorylation of EIF4E, EEF2, EEF2 kinase, and p70S6 kinase induced by high glucose levels, indicating that it affects important events in both initiation and elongation phases of mRNA translation (Lee et al. 2010). In the present study acetyl-EIF5A was found to be down-regulated by Resv treatment. Acetylation of EIF5A has been seldom reported or its function discussed. Lee et al. (2010) reported that the hypusine of EIF5A could be acetylated by spermidine/spermine acetyltransferase 1 (SSAT1), which resulted in its inactivation and a change in intracellular localization of the protein species (Lee et al. 2009, 2011). Our data in Fig. 5 show that Resv can down-regulate the level of total EEF2 starting at low concentrations, and up-regulate phospho-EEF2 at high concentrations. Since EEF2 and acetyl-EIF5A are the most significantly affected protein species identified in the present study and taking into account their essential roles in protein synthesis and connection with aging, their dysregulation must be connected with the pleiotropic biological functions influenced by Resv. More interestingly, through drug affinity responsive target stability (DARTS), EEF4A was reported to be a molecular target for Resv (Lomenick et al. 2009).

Another protein species found to be altered by Resv treatment is Nicotinate-nucleotide pyrophosphorylase, also known as quinolinate phosphoribosyltransferase (QPRT). QPRT is a key enzyme in the de novo synthesis of Nicotinamide adenine dinucleotide (NAD^+) (Ishidoh et al. 2010). NAD^+ is well known as a crucial cofactor in the redox balance; it is involved in stress resistance which in turn affects longevity (Braidy et al. 2011; de Figueiredo et al. 2011). The sirtuin (SIRT) family is composed of NAD^+-dependent protein deacetylases, including SIRT1, which play important roles in various fundamental biology processes (Yamamoto et al. 2007; Wood et al. 2004). Sirtuins also play important roles in cardiovascular disease. By modulating the activity of endothelial nitric oxide synthase (eNOS), of FoxO1, and of p53, and of angiotensin II type 1 receptor (AT1R), SIRT1 also promotes vasodilatory and regenerative functions in endothelial and smooth muscle cells of the vascular wall (Borradaile and Pickering

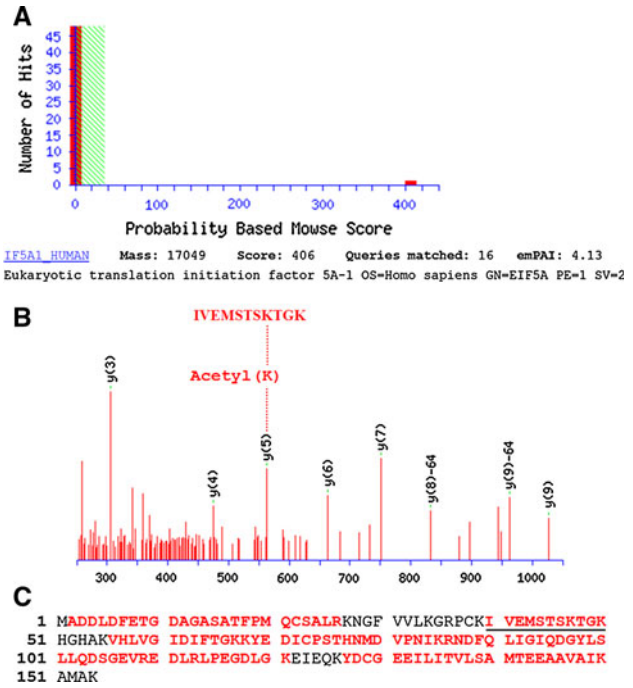

Fig. 4 Representative MS/MS Identification of acetyl-EIF5A (spot # 3). (**a**) Database searching with the MASCOT program using MS/MS data resulted in the identification of acetyl-EIF5A; (**b**) Representative MS/MS spectrum of acetyl-peptide (IVEMSTSKTGK); (**c**) Protein sequence of EIF5A, with the matched peptides shown in *red*, and the identified acetyl-peptide (IVEMSTSKTGK) *underlined*

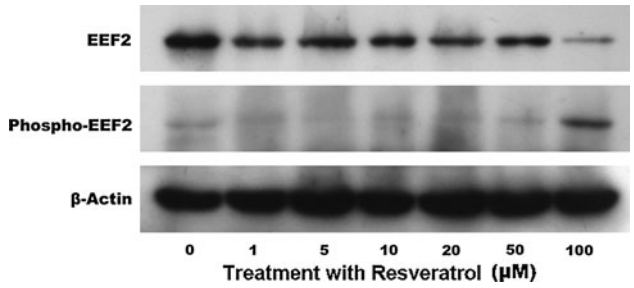

Fig. 5 Immunoblot analysis of the level of EEF2 and phosphor-EEF2 in response to resveratrol treatment. Resveratrol treatment (48 h) can down-regulate the level of total EEF2 even at low concentration (1 μM), and up-regulates phospho-EEF2 (T56) only at high concentration (100 μM). Similar results were obtained in at least three independent experiments

2009a, b). Because of the potentially beneficial effects of SIRT activation on cardiovascular health, these enzymes have been suggested to be valuable pharmaceutical targets for managing aging-associated diseases including cardiovascular disease, and interest in developing specific SIRT agonists is increasing (Chen and Guarente 2007). Resv has been reported to activate SIRT1, and many beneficial effects of Resv are thought to be produced through the activation of SIRT1 (Dolinsky and Dyck 2011; Baur et al. 2006; Lagouge et al. 2006; Wood et al. 2004), although it was reported that Resv is not a direct activator of SIRT1 (Pacholec et al. 2010; Beher et al. 2009). It is known that SIRT activity depends on cellular NAD^+ availability, so QPRT, a key enzyme in the de novo synthesis of NAD^+, may also be a potential pharmaceutical target to interfere on SIRT1 activity.

Other protein species identified include carboxymethyl-CFL1, BANF1, phospho-HSP27, HSP27, phospho-STMN1, and ADI1, whose functions are connected with diverse biological processes. Carboxymethyl-CFL1 is a widely distributed intracellular actin-modulating protein that binds and depolymerizes filamentous F-actin, and inhibits the polymerization of monomeric G-actin in a pH-dependent manner (McCullough et al. 2011). BANF1 was first identified by its ability to protect retroviruses from intramolecular integration and therefore promote intermolecular integration into the host cell genome. This protein is thought to facilitate nuclear reassembly by binding with both DNA and inner nuclear membrane protein species and thereby recruit chromatin to the nuclear periphery (Bradley et al. 2005; Van Maele et al. 2006; Capanni et al. 2010). HSP27 and its phosphorylated protein species are multifunctional molecules associated with various biological processes including cell cycle, growth, apoptosis, and aging (Doshi et al. 2010; Kostenko and Moens 2009; Ferns et al. 2006). STMN1 encodes a ubiquitous cytosolic phosphoprotein and was proposed to be a small regulatory protein and a relay integrating diverse intracellular signaling pathways involved in the control of cell proliferation and differentiation. STMN1 was reported to be involved in the regulation of the microtubule filament system by destabilizing microtubules (Curmi et al. 1999; Rubin and Atweh 2004). ADI1 is an enzyme that belongs to the acireductone dioxygenase family of metal-binding enzymes, which are involved in methionine salvage. ADI1 may regulate mRNA processing in the nucleus and may carry out different functions depending on its localization (Gotoh et al. 2007).

It is notable that several protein species with post-transcriptional modification (PTM) (carboxymethyl-, acetyl-, and phospho-) were found to be altered following exposure to Resv. These results demonstrate that PTM play important roles in cell biology processes and also proved that 2DE–MS/MS proteomic tools are valuable in the identification of variations in protein PTM. For example, carboxymethyl-CFL1 and acetyl-EIF5A have rarely been reported by other groups. Proteins with different PTM have been suggested to be identified as different protein species, a term firstly defined by Jungblut et al. (2008).

Recently, Resv has been shown to mediate some of its effects through inhibition of cyclooxygenases (COX) (Baur et al. 2006; Pacholec et al. 2010; Lagouge et al. 2006;

Beher et al. 2009; Wood et al. 2004; Barger et al. 2008; Zhang 2006). COX is an enzyme that is responsible for the formation of such important biological mediators as the prostanoids and hence plays an important role in inflammation (Rouzer and Marnett 2009). It was also reported that Resv can attenuate mitochondrial oxidative stress and induce mitochondrial biogenesis in endothelial cells (Ungvari et al. 2009; Csiszar et al. 2009). The nine protein species identified in the present study have not been reported to be functionally related to COX pathway and mitochondrial biogenesis process in any direct way.

Future perspectives

In the present study, using proteomic tools nine protein species were found to be altered in HUVECs following resveratrol treatment. Further studies will be needed to answer two important questions, namely whether EEF2 and EIF5A are the direct binding targets of Resv and whether Resv activates SIRT1 mainly through the up-regulation of QPRT. At the same time, additional studies need to be conducted to elucidate the possible crosstalk among the affected protein species and their potential relationship to the COX pathway, and to the mitochondrial biogenesis processes, which have been shown to play important roles in the pleiotropic effects of Resv.

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