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Salidroside influences the cellular cross-talk of human fetal lung diploid fibroblasts: A proteomic approach

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ABSTRACT

Senescence is a complex multiple factor proces, which is still poorly understood. The purpose of this study was to find the proteome of cultured human fetal lung diploid fibroblasts (2BS) of different population doubling (PD), as well as the altered proteome induced by salidroside (SAL) in 2BS cells. Proteins were identified by two-dimensional electrophoresis (2-DE) combining matrix-assisted laser desorption/ionization-time and flight mass spectrometry (MAL DI-TOF/MS). As a result, we found 16 proteins with two-fold variations in senescent cells or after SAL treatment, some being reduced such as reticulocalbin-1, heat shock protein beta-6, elongation factor 1-delta, F-actin-capping protein subunit alpha-1, and chloride intracellular channel 1. In contrast, 40S ribosomal protein SA, proteasome subunit alpha type-5, and zinc finger BED domain-containing protein 5 increased with cell age. Furthermore, heat shock protein beta-6, Zinc finger BED domain-containing protein 5 was increased in PD30 cells after 10 μM SAL treatment, whereas, elongation factor 1-delta, 6-phosphogluconolactonase, Nucleoside diphosphate kinase A, F-actin-capping protein subunit alpha-1, Probable ATP-dependent RNA helicase DDX41, Chloride intracellular channel 1, and Peroxiredoxin-6 were increased in PD50 cells after 10 μM SAL treatment. Some of these proteins were involved in the protein synthetic and degradative pathways, which emphasizes the metabolic disorder or functional impairment of cell senescence. Moreover, these proteins could be candidate biomarkers for evaluating the SAL anti-senescence effect.

1. Introduction

Aging is a complicated process and ultimately leads to morbidity. In the current world's population, the aging population(> 60 years old) is around 11% and it will reach ~22% in 2020 (Kour and Rath, 2016). Aging is believed to be the major risk factor for various diseases, such as cardiovascular malfunctions, neurological disorders, metabolic disruptions and so on. Senescence, accompanied by some main functional alterations, such as β-Gal positive, irregular cell, is the foundation of organism aging (Dimri et al., 1995). And now, the senescence process has greatly contributed to hospitalization or death among old people, so much more effort has been made to develop anti-aging agents that attenuate cellular senescence (Sikora et al., 2011). Salidroside (SAL) (Supplymental Fig. 1) is a glucoside of tyrosol in Rhodiola rosea L and it is one of the major compounds that participate in a series of pathological processes such as endothelial dysfunction, immune regulation and osteoporosis (Yuan et al., 2013; Zhang et al., 2013a, b; Zhao et al., 2013). Our previous investigation showed that SAL is able to reduce the AGEs level and inhibits AGEs formation both in vitro and in vivo to contribute to its anti-aging effect aging model (Mao et al., 2010; Mao et al., 2012). Additionally, in the H_2O_2-induced senescent 2BS cells, the protective potential of SAL against senescence was also proved by determination of senescence associated proteins, such as P21, P16 (Mao et al., 2015). However, the associated protein biomarkers responsible for the anti-senescence property of SAL have not yet been fully elucidated in the literature.

Identification of the biomarkers could be supported by detailed characterization of 2BS cells through 2-DE and mass spectrometry to determine global expression profiles of proteins and the alteration protein profiles induced by SAL. Proteomics has become a standard tool in molecular biology to explore cellular mechanisms at the protein level. Two-dimensional gel electrophoresis approaches still offer the highest available resolution at the intact protein level, with the added benefit of including protein isoforms and posttranslational modifications in the global picture (Boraldi et al., 2007). Previous study performed in our laboratory allowed the creation of protein profile of

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normal human 2BS cells (Xing et al., 2015). Additionally, the proteomic approach has already been applied to study the occurrence of senescence in other cell types (Boraldi et al., 2007; Boraldi et al., 2003). Therefore, in our study we have attempted to analyze for the first time the changes that may occur during cellular senescence of human 2BS cells at the level of protein expression profiles and modification of proteins induced by SAL.

2. Materials and methods

2.1. Cells and treatments

As our previous word described, the human fetal lung diploid fibroblasts 2BS cell line was purchased from the National Institute of Biological Products (Beijing, China), and had been widely used as a cellular senescence model (Li et al., 1995; Tang et al., 1994). The cells are considered to be young at the population doubling (PD) ≤30 and fully senescent at PD ≥ 55. Cells were cultured in DMEM supplemented with 10% FBS, penicillin (100 U mL^{-1}) and streptomycin (100 mg L^{-1}) at 37 °C in a humidified atmosphere containing 5% CO_2. SAL was dissolved in dimethyl sulfoxide (DMSO) as a 10 mM stock solution. Confluent cultures of 2BS cells were split to a 2×10^6 cells/10 cm dish and cultured for 24 h. Then, the PD 30 cells and PD 50 were treated with a dosage of 10 μM SAL for 24 h. The control group was cultured with 0.1% DMSO for 24 h.

2.2. Proteome analysis

2.2.1. Sample preparation

The experiment was performed as previously described (Boraldi et al., 2003). Briefly, 2BS cells were grown for 24 h in DMEM plus 10% FBS with 10 μM SAL. Afterwards, cells were collected from the 10 cm dish through incubation in 0.25% trypsin. And then washed in DMEM + 10% FBS, proteinase inhibitors; then centrifuged at 1000*g* (4 °C) for 10 min. Next, the supernatant was removed and was re-suspended in PBS and proteinase inhibitors, and then centrifuged at 1000*g* (4 °C) for 10 min again, and then immediately resuspended in 300 μl of 8 M urea, 4% CHAPS, 40 mM Tris base, 65 mM dithioerythritol (DTE), and protein concentrations were determined by BCA protein assay kit (Pierce Chemical Co.), and then frozen at −80 °C until use. Cells from each group were kept separate during the whole experiment.

2.2.2. Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis of each sample was performed using the Immobiline/polyacrylamide system, essentially as described by (Boraldi et al., 2003). A quantity of 30 μg total protein was used for each electrophoretic run. Isoelectric focusing (IEF) was performed on an Ettan IPGphor 3 IEF system (GE Healthcare, USA) as follows: 30 V for 12 h, 500 V for 1 h, 1000 V for 1 h, 6400 V for 2 h, and finally 8000 V for 2 h. Then, the IPG strips were equilibrated in: (1) reducing buffer and alkylating buffer for 15 min.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in the Ettan Dalt twelve system (Amersham). The IPG strips were loaded onto gels for 45 min at 5 W and for 6 h at 20 W. Then, the gels were stained by silver nitrate as described by previous study (Shevchenko et al., 1996), which was then scanned by UMAX Image Scanner and analyzed using Image Master 2D Platinum software (GE Healthcare, USA).

2.2.3. Spots selection and analysis

All stained gels were digitalized by UMAX Image Scanner (GE, Healthcare, USA) and Image Master 2D Platinum software 6.0(GE Healthcare, USA) to find the significant different proteins between the SAL groups and DMSO groups. After background subtraction, each paired spot was edited manually. The volume of each spot was normalized against total spot volume, and the spot was thought to be significant if $P < 0.05$. These spots were then selected as candidate proteins for MALDI-TOF/MS analysis and subjected to in-gel tryptic digestion.

2.2.4. Peptide identification

All interest spots on the gels were selected and excised from the gels, and then processed by following these steps: (1) washing and destaining: i, destained: 100 μl of working solution(30 mM Potassium Ferricyanide ($K_3Fe(CN)_6$):100 mM Sodium Thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$) = 1:1), shaking until the brownish color is removed; ii, wash: 3 × 5 min with deionized water and 200 mM NH_4HCO_3 to remove the yellow reageant; iii, dehydrate: 200 μl 100%ACN, 10 min; iv, Dry: remove the ACN and dry in speed vacuum for 30 min (Gharahdaghi et al., 1999). (2) Reduction and Alkylation of cystein residues: i, reduce: 50 μl reduce solution (10 mM DTT in 100 mM NH_4HCO_3), 45 min, 56 °C; ii, alkylate: 50 μl alkylation solution (55 mM iodoacetamide in 100 mM NH_4HCO_3); iii,wash and dry following steps as above. (3) Enzymatic Digestion: i, digest: in 15 μl of 50 mM NH_4HCO_3, 5 μl of trypsin, on ice, 30 min; ii, replace: Remove remained enzyme supernatant, and replace it with 5 μl of 50 mM NH_4HCO_3 to keep the gel pieces wet during enzymatic cleavage; iii, digest: overnight at 37 °C. (4) Extraction of peptides: centrifuge and transfer the supernatant into another tube; 50 μl 50%ACN/0.1% TFA, sonificate for 3 × 1 min; spin and transfer the extracts to the primary supernatants.

2.2.5. MALDI-TOF MS protein identification

The tryptic peptide extracts were re-dissolved in 12 μl 0.1% TFA. For direct spotting onto MALDI-TOF/MS TARGET, elute with matrix in elution solution (α-HCCA fully dissolve in TA(0.1% TFA: ACN = 2:1)). Briefly, (1) pipette 0.5–4 μl of desalted-concentrated sample directly onto target by depressing plunger until appropriate volume is dispensed; (2) save or discard the remaining sample. Make the elution solution fresh daily. Saturate an aliquot of the following elution solution with a suitable crystallization matrix, votax the mixture for 60 s, and then centrifuge (at least 12,000 *g*) for 20 s. Take care to use only the supernatant as any particle in the solution can act as a nucleation site and will cause irregular or patchy crystallization. And then, the spectra was analyzed with MALDI-MS (4800 Proteomics Analyzer, Applied Biosystems, USA). And then, the peaks were searched by the MASCOT search engine (www.matrixscience.com), with a mass tolerance within 40 ppm in the NCBI databases. The UniProt Knowledgebase was used.

2.2.6. Western blot analysis

The experiment was performed as previously described (Guo et al., 2016). Briefly, 2BS cells at PD 50 pre-treated with SAL (10 μM) were harvested 24 h later. The cells were washed with iced PBS and then lysed with cell lysis buffer containing protease inhibitors cocktail (Cell Signaling Technology, USA). Protein concentrations were determined by BCA protein assay kit (Pierce Chemical Co). 20 μg protein was loaded on 10% SDS polyacrylamide gel (Bio-Rad, USA). The transferred membranes were cultured with EEF1D and PSMA5 monoclonal antibodies (Sigma, USA), respectively. The membrane was then incubated for another 2 h at room temperature with the secondary antibody, and β-actin was used as internal loading control. The protein bands were detected by the enhanced chemiluminescence (ECL) method, and the was quantified by ImageJ software.

2.2.7. Statistical analysis

Data is expressed as means ± SDs and analyzed with one-way ANOVA method by the GraphPad software program(5.0). $P < 0.05$ was considered significant.

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