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Exposure of cultured fibroblasts to the peptide PR-11 for the identification of induced proteome alterations and discovery of novel potential ligands

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ABSTRACT

The PR-11 peptide corresponds to the N-terminal and active region of the endogenously synthesized PR-39 molecule, of porcine origin. It is known to possess various biological effects including antimicrobial properties, angiogenic and anti-inflammatory activities. Apart from its reported activity as a proteasome inhibitor, a more comprehensive understanding of its function, at the molecular level, is still lacking. In this study, we used a label-free shotgun strategy to evaluate the proteomic alterations caused by exposure of cultured fibroblasts to the peptide PR-11. This approach revealed that more than half of the identified molecules were related to signalling, transcription and translation. Proteins directly associated to regulation of angiogenesis and interaction with the hypoxia-inducible factor 1-α (HIF-1α) were significantly altered. In addition, at least three differentially expressed molecules of the NF-κB pathway were detected, suggesting an anti-inflammatory property of PR-11. At last, we demonstrated novel potential ligands of PR-11, through its immobilization for affinity chromatography. Among the eluted molecules, gC1qR, a known complement receptor, appeared markedly enriched. This provided preliminary evidence of a PR-11 ligand possibly involved in the internalization of this peptide. Altogether, our findings contributed to a better understanding of the cellular pathways affected by PR-39 derived molecules.

1. Introduction

The peptide PR-11, a ~1.4 kDa molecule highly rich in proline and arginine residues, corresponds to the N-terminal active region of PR-39, the latter formerly isolated from the small intestine [1] and bone marrow of pigs [2]. PR-39 acts against a wide spectrum of bacteria, including clinical isolates resistant to multiple drugs [3,4]. At micromolar concentrations, the peptide is rapidly internalized and interferes with various cellular processes such as inhibition of DNA synthesis and translation. At higher levels, a bactericide activity is also observed, possibly by perturbation of cell membrane stability [5,6]. Aside from its antimicrobial properties, PR-39 stimulates neutrophil migration [7], inhibits apoptosis [8–10] and reduces motility and cell proliferation in cancer tissues [11,12].

PR-39 has been shown to bind intracellular SH3 domain-containing proteins [13] and its role as a proteasome regulator also reported. The hypoxia signalling and NF-κB pathways are known to be compromised by PR-39 proteasome-dependent inhibition resulting in angiogenic [14] and anti-inflammatory effects [15,16], respectively. Sequential C-terminal residue deletions of PR-39 in parallel to evaluation of the resulting activity over the 20S proteasome, revealed the requirement of at least 11 N-terminal amino acids to sustain its inhibitory property, in a dose-dependent manner [17]. Atomic force microscopy also demonstrated that upon binding of PR-39 and PR-11 to 26S and 20S proteasomes, their cylindrical architecture is reversibly altered [17].

Given the wide repertoire of biological activities of PR-39 and PR-11 and the limited knowledge of their interfering molecular pathways, we used a label-free shotgun approach to evaluate the proteome alterations caused by exposure of cultured fibroblasts to 1 μM PR-11. Our findings demonstrated that >50% of the identified differentially expressed proteins are related to cell signalling, transcription and translation. In addition, using immobilized PR-11 affinity chromatography, we were

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able to identify novel potential ligands, providing a better understanding of its mechanism of action.

2. Materials and methods

2.1. Ethics statement

The procedures involving animals were carried out in accordance with the Brazilian legislation (11790/2008). They were reviewed and approved by the local ethics committee on animal experimentation (CEUA), Universidade Federal de Ouro Preto (UFOP), and received the protocol number 2013/09.

2.2. Synthesis of the PR-11 peptide

PR-11 peptide was chemically synthesized based on the amino acid composition of the 11 N-terminal residues (RRRPRPPYLPR) of PR-39. PR-11 was purified by HPLC using reversed-phase chromatography (Shimadzu Scientific Instruments) (Supplementary Fig. 1), identified by direct injection in an IT-TOF mass spectrometer (Shimadzu Scientific Instruments) and finally reconstituted in water. Peptide concentration was calculated using the molar extinction coefficient at 280 nm of its constituent tyrosine residue (1280 M^{-1} cm^{-1}).

2.3. Fibroblasts culture and exposure to PR-11 peptide

Fibroblasts were obtained from lungs of neonate Wistar rats, aged 2 days and of approximately 5 g weight. Briefly, after removal of the lungs, these were washed in 1× ADS buffer (115 mM NaCl; 20 mM Hepes, 1 mM Na_2HPO_4, 5 mM D-glucose; 5 mM KCl; 1.6 mM $MgSO_4$) and subjected to 3 cycles of digestion with 0.8 mg/mL pancreatin (Sigma-Aldrich) for 20 min at 37 °C. Cells were recovered by centrifugation at 1.000 ×*g* for 10 min and resuspended in DMEM medium supplemented with 15% v/v fetal bovine serum and 1% v/v penicillin/streptomycin. Prior to exposure of cells to PR-11, a minimum cell confluence of >90% throughout the well was required under microscopic observation and the supernatant should contain a negligible number of detached cells. Primary fibroblast cultures (unique passage) were exposed to 1 μM PR-11 during 2, 6 and 10 h. Control cultures, in which water was added instead of the water-soluble peptide, were obtained for the same time points. At the end of the incubation periods, the supernatant containing PR-11 peptide was completely removed and the cells were gently detached from the wells using the TrypLE reagent (Gibco). These were finally recovered by centrifugation. The experiments were performed in biological triplicates.

2.4. Soluble protein extract and in solution digestion

Control and treated fibroblasts were resuspended in 500 μL of 25 mM Tris-HCl pH 7.5; 1 mM DTT and 1% v/v glycerol buffer containing 1× protease inhibitor cocktail (Sigma-Aldrich). Samples were sonicated on ice through 4 cycles of 20 pulses each, with 45 s rest between cycles. The homogenates were centrifuged at 100,000 ×*g* for 1 h and the protein concentration determined by BCA method (Thermo Scientific).

Soluble proteins present in a 20 μg aliquot were reduced using 4 mM dithiothreitol (Sigma-Aldrich) in 100 mM ammonium bicarbonate at 56 °C for 15 min and then alkylated in 8 mM iodoacetamide (Sigma-Aldrich) for 15 min in the dark. Enzymatic digestion was carried out at 37 °C for 18 h using 0.8 μg Sequencing Grade Modified Trypsin (Promega) and the reaction was interrupted by acidification with 10 μL acetic acid. Tryptic peptides were cleaned up using a Strata C18-E cartridge (55 μm, Phenomenex), dried over speed vacuum and resuspended in 0.1% v/v formic acid.

2.5. Mass spectrometry analysis: in solution digestion

For each sample, 3 μg of tryptic peptides were separated in a UltiMate® 3000 UHPLC system (Thermo Scientific) equipped with a C18 column (PepMap Acclaim RSLC — 75 nm × 15 cm, Thermo Scientific) under mobile phase flow of 0.3 μL/min using a nonlinear gradient (4 to 90% of 80% *v*/v acetonitrile and 0.1% v/v formic acid) during 180 min. The eluted peptides were ionized in a ESI-nanospray interface and analyzed in a Q-Exactive™ Hybrid Quadrupole-Orbitrap instrument (Thermo Scientific) under the acquisition mode Full MS followed by MS/MS. The following operating parameters were set: Full MS resolution: 70.000; MS/MS resolution: 17,500; scan range: 300–2000 *m*/*z*; 12 most abundant isotope patterns scanned; loop count: 10; isolation window: 2.0 *m*/*z*; ions exhibiting charge +2, +3 or +4; dynamic exclusion: 60 s; positive ionization mode.

The Xcalibur v.3.0.63.3 and MaxQuant v.1.5.2.8 softwares [18] were used for the acquisition and data analysis, respectively. Database searches were performed using a UniProt *Rattus norvegicus* compilation containing 30,091 sequences. Search parameters included: enzyme: trypsin/P; carbamidomethylation of cysteine as fixed modification; oxidation of methionine and N-terminal acetylation as variable; maximum missed cleavage sites: 2; mass tolerance: 4.5 ppm; isotope match tolerance: 2 ppm; minimum peak length: 2; False Discovery Rate (FDR) and Peptide Sequence Match (PSM): 0.01; minimum ratio count: 2. Relative abundance of proteins were obtained using Label-Free Quantification (LFQ) provided by the LFQ intensity data (unique + razor peptides).

2.6. Statistical analyses and protein functional categorization

Statistical analysis was performed using the Graph Pad Prism software v.6.01. For each exposure time to the PR-11 peptide, proteins exhibiting at least two LFQ intensity data among the three biological triplicates were regarded genuine identifications. These were subjected to a *t*-test and the proteins with $p < 0.01$ were considered significantly altered. Differentially expressed proteins were categorized using the UniProtKB database (available at www.uniprot.org) according to their biological functions.

2.7. Total protein extraction, affinity chromatography and in gel digestion

A liver protein extract from Wistar rat was obtained for use in immobilized PR-11 affinity chromatography. Approximately 100 mg tissue section was homogeneized in 1 mL of extraction buffer (50 mM Tris-HCl pH 7.5; 100 mM NaCl) containing 1× protease inhibitor cocktail (Sigma-Aldrich). Sample was sonicated on ice through 5 cycles of 20 pulses each, with 45 s rest between cycles. The homogenate was centrifuged at 20,000 ×*g* for 1 h and the protein concentration determined by BCA method (Thermo Scientific).

Coupling of PR-11 peptide to the Sepharose 4B matrix was performed as previously described [19]. Approximately 10 mg of total proteins were loaded onto a 1 mL column containing immobilized PR-11. The column was extensively washed with 50 mM Tris-HCl pH 7.5; 300 mM NaCl and 5 mM $MgCl_2$ and the bound fraction recovered after loading 1 mL of 50 μM PR-11. The collected samples were dialyzed in 10 mM ammonium acetate pH 7.4 and dried over speed vacuum. Aliquots taken from the collected samples were analyzed under denaturing conditions using 12% SDS-PAGE as classically described [20] and the gel stained in silver nitrate.

Visualized bands from the bound fraction were excised manually for in gel digestion. The bands were destained in 0.5% w/v potassium ferricyanide/10% w/v sodium thiosulfate and washed in 40% v/v ethanol/7% v/v acetic acid. Disulfide bonds were reduced in 500 μL of 50 mM DTT at 65 °C for 30 min and alkylated in 300 μL of 100 mM iodoacetamide at room temperature for 1 h in the dark. Gel pieces were washed in 500 μL of 20 mM NH_4HCO_3/50% v/v acetonitrile for 3 × 20 min and dried in a speed vacuum. Then, gel pieces were rehydrated in 20 μL of