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The changes of Proteome in MG-63 cells after induced by calcitonin gene-related peptide

Meng Wu [a], Zhiliang Zhao [b], Yan Han [a,*]

[a] Department of Plastic Surgery, General Hospital of PLA, Beijing 100853, PR China
[b] Wound Healing and Cell Biology Laboratory, Institute of Basic Medical Sciences, General Hospital of PLA, Beijing 100853, PR China

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ABSTRACT

The aim of this study was to elucidate the changes of Proteomics in MG-63 cells after induced by calcitonin gene-related peptide (CGRP). The subcultured MG63 cells were randomly divided into CGRP group and the control group. Two-dimensional electrophoresis (2-DE), coomassie brilliant blue gel staining and mass spectrometry were used to analyze the changes of protein extracted from the two groups. Six protein spots with significant differences were selected to take in-gel digestion, peptide mass fingerprint analysis and IPI database search. There were more than 967 ± 17 protein spots separated by Two-dimensional gel electrophoresis and the matching rate was (85.1 ± 1.4%). Compared with the control group, six protein spots were significantly different in CGRP-induced group and expression of the 6 differently proteins was downregulated. Mass spectrometry analysis identified 5 proteins, including ribosome binding protein p180 which is related to new synthetic protein's translocation and calcium-binding protein (HRNR Hornerin) that can affect intracellular calcium concentration to regulate cell activity. These changes of proteome, including several groups of proteins that influence calcium ion concentration and new protein translocation, suggested that CGRP may regulate the cells by the second messengers and cytokines. This study contributes to a better understanding of the molecular mechanisms in CGRP-induced MG-63 cells.

1. Introduction

Calcitonin gene-related peptide (CGRP) has a broad distribution and expression pattern in vivo. It is transcribed from the same gene locus as calcitonin (CT). CGRP plays a significant pathophysiological role in the regulation of bone metabolism, the cardiovascular system, the gastrointestinal system and pain [1]. CGRP is one of the most widely distributed sensory neuropeptides in bone tissue and is largely present in areas undergoing active bone metabolism. During bone wound healing, CGRP and other neuropeptide substances are secreted by nerve endings in the bony callus and promote bone wound repair and reconstruction [2]. It can promote the proliferation and differentiation of osteoblasts [3,4]. Our previous experiments have shown its importance in jaw wound healing [5]. CGRP has also been demonstrated to control nitric oxide production in osteoblasts in vitro [6]. MG-63 cells were cultured from a human osteosarcoma and immortalised to generate an osteoblast-like cell line. Bone wound healing studies similar to ours often use MG-63 cells as an experimental model [7,8].

In our study, we aim to find the protein which interact with CGRP and play an important role in the process of fracture healing. Analyzed by two-dimensional electrophoresis (2-DE) and mass spectrometry and studying changes of the overall protein level of osteoblast cell after treatment with CGRP, we determined their relationship in the upstream and downstream signaling pathways so as to explore the mechanism of action of CGRP in the fracture healing process.

2. Materials and methods

2.1. Materials

Human CGRP was purchased from Sigma–Aldrich Corp. (Sigma, St. Louis, MO). CGRP was dissolved in distilled water to a stock concentration of 100 μM and stored in 100 μl aliquots at −80 °C. The

Abbreviations: CGRP, calcitonin gene-related peptide; 2-DE, two-dimensional electrophoresis; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; IEF, isoelectronic focusing; min, minutes.

* Corresponding author.
E-mail addresses: ppxixi@163.com (M. Wu), zzl81@hotmail.com (Z. Zhao), hanyan_301@qq.com (Y. Han).

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following reagents were purchased from Invitrogen Corp. (Invitrogen, Carlsbad, CA): ethylene laminate triacetin acid (EDTA), phenylmethylsulfonyl fluoride (PMSF), sodium dodecylsulphate (SDS), Opti-MEM® Reduced Serum Medium and TRIzol® Reagent. RAMP1 (FL-148) and CRLR (V-20) antibodies were purchased from Santa Cruz Biotechnology. The Reverse Transcription System was purchased from Promega Corp. (Promega, Madison, WI, USA).

2.2. Cell culture

MG-63 human osteoblast-like cells were obtained from ATCC (America Type Culture Collection). Cells were cultured in DMEM (Dulbecco's modified Eagle's medium; Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (GIBCO, Grand Island, NY), 100 U/ml penicillin and 0.1 mg/ml streptomycin. Cells were subcultured every 72 h in a humidified 5% CO_2 incubator. Cells from passages 4–6 were used in the experiments.

When cells were cultured until logarithmic growth phase, in which growth density is more than 80%, we replaced with serum-free medium for 24 h (synchronous processing) and then randomly divided them into two groups: CGRP group, CGRP was dissolved in serum-free DMEM medium and added into cell culture flasks where final concentration was maintained at 10^{-7} to 10^{-10}mol /L. Incubator was set at 37 °C, 5% CO_2. Control group, the same amount of physiological saline were added to cell culture flasks, cultured under the same experimental conditions.

2.3. Protein extraction

Cells were washed three times with ice cold PBS and then incubated with lysis buffer (20 mM HEPES, pH 7.5, 200 mM NaCl, 0.2 mM EDTA–Na_2, 1% Triton X-100, 0.05% SDS, 0.5 mM DTT, 1 mM Na_3VO_4, 20 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin and 10 mg/ml leupeptin) at 48 °C for 30 min and centrifuged at 12,000*g* for 5 min; the supernatant was stored at −80 °C. The protein concentration of the supernatant was determined by the 2D-Quant Kit (Amersham Bioscience, Uppsala, Sweden).

2.4. Two-dimensional gel electrophoresis

Isoelectronic focusing (IEF) was performed with 18-cm immobilized strips with pH value of 3–10 and 4–9 in an IPG phor electrophoresis device (Amersham Bioscience). Samples (1 mg for Coomassie staining, 300 mg for silver staining) were diluted into 350 mL of a buffer containing 7 M of urea, 2 M of thiourea, 4% CHAPS, 0.5% IPG buffer at pH 3-10, 65 mM 1,4-dithioerythritol, and traces of bromophenol blue and loaded onto an IPG strip. The IEF was carried out at 20 °C under the following schedule: 12 h at 30 V, 2 h at 100 V, 1 h at 500 V, 1 h at 1000 V, 1 h of a linear gradient to 8000 V, and 10 h at 8000 V. After IEF, the strips were equilibrated first in 6 M of urea, 30% glycerol, 2% sodium dodecyl sulfate (SDS), 50 mM Tris–HCl (pH 6.8) containing 1% 1,4-dithiothreitol (DTT) for 15 min in the first equilibration step and then in 2.5% (w/v) iodoacetamide in the second step. The strips were transferred onto the 2DE gel, sealed in place with 1% agarose. The two-dimensional SDS–polyacrylamide gel electrophoresis (PAGE) was performed on 12% polyacrylamide gels at 20 mA and 16 V on Bio-Rad Protein II (Bio-Rad Hercules, CA) and was terminated when the bromophenol blue front had migrated to the lower end of the gels.

2.5. Gel staining and image analysis

The gels were stained either with Coomassie brilliant blue R-350 (Amersham Bioscience) or silver staining. The silver staining method is compatible with protein identification using mass spectrometry as described by Shevchenko and co-workers [9]. Gels were scanned with the ImageScanner (Amersham Pharmacia, Piscataway, NJ) and analyzed with ImageMaster 2-D Elite software (Amersham Pharmacia). The software converts the signal intensity of a spot in the gels into digital data described as volume value. For normalization, the volume of each spot on a gel was divided by the total volume of all spots and spot intensities were expressed as percentage of the total sum of spot volumes. Spot intensity ratio of matched spots in the experimental gels (adenomyosis) and control gels (normal uterine muscle) were computed. The protein spots with intensity ratio more than two were considered dysregulated and were identified using mass spectrometry.

2.6. Tryptic digestion of two-dimensional gel spots

Coomassie-stained spots were manually excised and destained with 100 ml of 50% acetonitrile in 50 mmol/L ammonium bicarbonate. The liquid phase was removed and the gel spots were completely dried in a SpeedVac vacuum concentration system (Savant, New York, NY). Then the dried gel pieces were digested with 10 mL of 12.5 ng/mL trypsin (Roche, Rotkreuz, Switzerland) in 50 mmol/L ammonium bicarbonate at 37 °C overnight. The silver-stained spots were destained with 50 mL of 30 mmol/LK_3-$Fe(CN)_6$ and 100 mmol/$LNa_2S_2O_3$ for 2 min. The spots were washed with ultrapure water, dehydrated with 100% acetonitrile, and dried in a SpeedVac. Pieces of each spot were reduced by incubation in 50 mL of 10 mmol/L dithiothreitol in 100 mmol/L ammonium bicarbonate at 57 °C for 1 h. The pieces were further alkylated by incubation in 50 mL of 55 mmol/L iodoacetamine in 100 mmol/L ammonium bicarbonate in a dark room at room temperature for 30 min. Then the gel pieces were washed with 100 mmol/L ammonium bicarbonate twice and dehydrated with 100% acetonitrile and dried in a SpeedVac. The dried gel pieces were digested with 5 mL of 12.5% trypsin in 50 mmol/L ammonium bicarbonate at 37 °C overnight. The peptides of each spot were extracted from the gel by incubation twice with 50% acetonitrile containing 5% trifluoroacetic acid and once with 50% acetonitrile containing 2.5% trifluoroacetic acid. The supernants obtained from the two steps were pooled and dried by SpeedVac and stored at −20 °C until analyzed.

2.7. Protein identification

The peptides extracted from each spot were dissolved in 0.1% trifluoroacetic acid and loaded on a $Ziptip_{c18}$ (Millipore, Bedford, MA), which had been washed with 50% acetonitrile containing 0.1% trifluoroacetic acid and equilibrated with 0.1% trifluoroacetic acid several times. The $Ziptip_{c18}$ was washed with 0.1% trifluoroacetic acid several times. The peptide sample was eluted from the $Ziptip_{c18}$ with a saturated solution of α-cyano-4-hydroxycinnamic acid in 70% acetonitrile and 0.1% trifluoroacetic acid and spotted on the target plate. Analysis of peptide masses was performed using MALDI-TOF-MS (Voyager DE PRO Biospectrometry Workstation, Applied Biosystems, Foster City, CA) with a 337-nm nitrogen laser and delayed extraction and operated in reflector mode. The positive ionization mode was used with an acceleration voltage of 20 kV and reflection voltage of 23 kV. Mass spectra were generated with the mass range of 800–3500 Da from every 300 shots. The spectra were internally calibrated using the autolysis product of trypsin (m/z: 1154, 2163).

2.8. Database search

A database search was performed using the MASCOT search engine (http://www.matrixscience.com) in the SWISS-PROT (http://www.expasy.ch/sport/) and the NCBI (http://www.

ncbi.nlm.nih.gov/Web/Search/index.html) database. Monoisotopic masses were used and search conditions were set as species (human), mass tolerance (100 ppm), maximum missed cleavages by trypsin (up to 1), and cysteine modification by carbamidomethylation.

3. Results

3.1. Protein expression profiling

The 2DE protein separation was performed on both pH 3-10 and 4-9 IPG strips. Staining with both Coomassie blue and silver was used to visualize the protein spots. The proteins were evenly distributed with an isoelectric point range of 5–12 and a molecular range of 14–200 kDa. Fig. 1 displays typical 2DE gel images of both CGRP-induced group cells and control group cells stained with Coomassie blue and silver. In the Coomassie blue-stained images, there were more than 967 ± 17 protein spots detected with the ImageMaster software (Amersham Pharmacia). With silver staining, about 50% more spots could be visualized. Some proteins were a train of spots indicating that isoforms or modified proteins were present and well separated by 2-DE.

3.2. Protein identification

We chose the protein spots of which expression differences were 1.8 times greater than that of the control group to take analysis, but failed to detect any protein pattern unique to stage of cycle. We found that the expression of six protein spots of CGRP-induced MG-63 cells showed significant differences when compared with the control group cells. The expression of four protein spots of CGRP-induced MG-63 cells were disappeared. The expression of two protein spots of CGRP-induced MG-63 cells were down-regulated. The changes of isoelectric points and relative molecular mass between the two groups were showed in Table 1.

The protein spots were subjected to in-gel digestion, MALDI-TOF mass measurement and database search based on peptide mass matching. Fig. 1 shows the observed peptide mass map of protein spot. Of the 6 differently expressed protein spots, 5 were successfully identified. Table 2 is a summary of the identified proteins. The number of peptides and sequence coverage as well as theoretical isoelectric point and molecular weight are showed. Most of the matched proteins have high sequence coverage and the matching peptides are more than five. The observed molecular weight and isoelectric point value for each protein spot can be calculated with ImageMaster software (Amersham Pharmacia). Candidates provided by the database search with the molecular weight and isoelectric point value close to the theoretical were considered.

Table 1
Proteomic changes in MG-63 cells induced by CGRP.

Number	Isoelectric point	Relative molecular mass	Volume of control group volume	Volume of CGRP-induced group
1	9.20	171.72	116.00	Disappeared
2	9.96	198.66	77.50	Disappeared
3	12.14	15.62	152.00	Disappeared
4	8.06	14.41	263.00	92.60
5	5.71	46.07	284.00	141.00
6	8.48	158.77	295.00	Disappeared

4. Discussion

Proteomics is a powerful technique for studying the protein profile of diseased tissue and normal tissue. It is a promising approach for discovering disease biomarkers and key proteins in pathogenesis [10]. Two-dimensional electrophoresis is the only technique that can separate complex mixtures of proteins, such as cell lysates, according to isoelectric point and molecular mass [11]. The proteomic expression map generated from 2-DE reflects changes in protein abundance, isoforms and post-translational modifications. When combined with mass spectrometry and bioinformatics, identification of these protein spots can be made

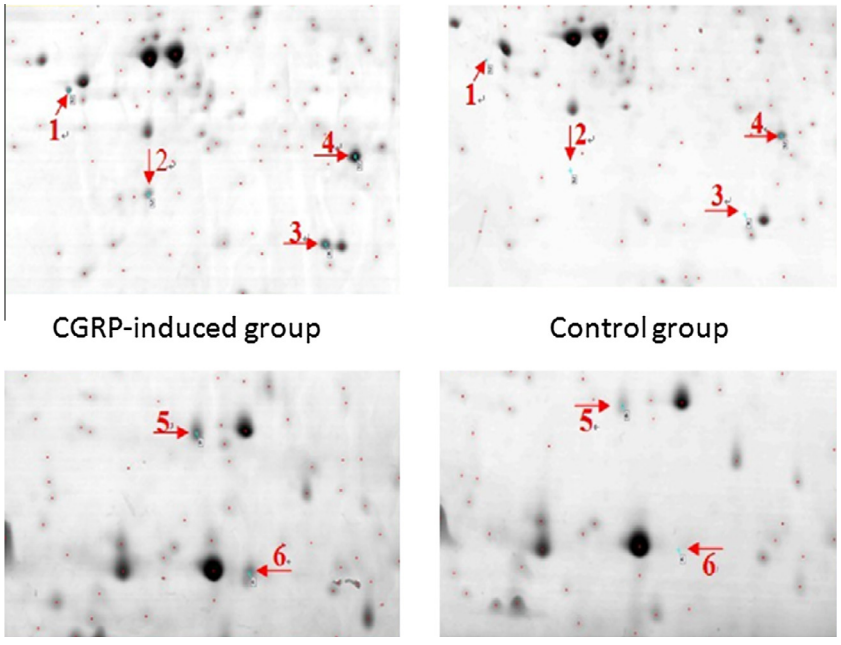

Fig. 1. Representative two-dimensional gel images of CGRP-induced group and control group samples. The proteins were separated on pH 4-9 IPG strips followed by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel. The separated proteins were detected by Coomassie blue in the upper two images and silver in the lower images. Marked numbers are dysregulated protein spots and identified according to Tables 1 and 2. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
The retrieval results in Spectrum Mill after mass spectrometry.

Spot number	Accession number	Sequence coverage	AA%	PI	MW	Protein identified
2	IPI00856098	75.51	9	9.00	165717.2	p180/Ribosome receptor
3	IPI00012880	16.28	16	11.12	12917.7	GNRH 2 Isoform 1 of Progonadoliberin-2 precursor
4	IPI00887022	57.70	6	9.84	190,565	HRNR Hornerin
5	IPI00021428	81.13	15	5.23	42051.3	ACTA1 Actin, alpha skeletal muscle
6	IPI00219219	10.74	8	5.34	14715.8	LGALS 1 Galectin-1

[12–14]. This technique may help discover the mechanism of endometriosis and give us a new methodology for further research.

In this paper, we adopt 2-DE (two-dimensional electrophoresis) to study the changes of Proteomics of CGRP-induced MG-63 cells. We identified five proteins with significant differences by mass spectrometry technology. (1) Ribosome binding protein (p180 (p180/ribosome receptor)) which is related to ribosomal localization and new synthetic protein's translocation [15,16]. (2) Calcium-binding protein (HRNR Hornerin) that can affect intracellular calcium concentration to regulate cell activity [17,18]. (3) Secretory protein (GNRH2 Isoform 1 of Progonadoliberin-2 precursor) [19,20]. (4) ACTA1 Actin (alpha skeletal muscle), which participate in cell morphology and various intracellular movement [21,22]. (5) Lectin ((LGALS 1 Galectin-1)), that can modify cells binding with extracellular matrix and other cells [23,24].

Literature review helps us understand the mechanism through which CGRP plays a role in fracture healing after its effect on osteoblasts [25]. Ca^{2+} plays a very important role in the regulation of the biological activity of the cells. Normal cells have a very low cytoplasmic Ca^{2+} concentration. When the cells receive their exogenous signals or experience metabolic abnormality, Ca^{2+} outside and inside the cell will soon enter cytoplasm and combine with Ca^{2+}-binding protein to form a second messenger and then play a series of biochemical reactions. Ca^{2+} and calmodulin complex can also activate other second messengers such as CAMP and CGMP, enhancing cell anabolism [26].

CGRP can regulate Ca^{2+} concentration in osteoblasts and thus can change osteoblast activity. But this effect varies with different extracellular environment and sometimes the effect is completely opposite. Drissi discovers that CGRP can induce a fast, temporary, dose-dependent cell sap Ca^{2+} concentration increase in OHS-4 osteoblast [27]. The level of intracellular Ca^{2+} is affected by many cytokines and other ion concentration. Out study shows HRNR Hornerin expression decrease probably because CGRP influence calcium ion concentration through HRNR Hornerin, a calcium ion binding protein, and then regulate cell activity.

LGALS1 Galectin-1 is a lectin that can modify cells combine with other cells or extracellular matrix. It can act as an autocrine negative regulator and can regulate cell proliferation. It may be involved in the bone healing process [28]. Ribosome binding protein p180 is related to ribosomal location and new synthetic protein's translocation [29]. It probably combines with newly synthesized CGRP in cells. This is non-functional binding.

In our experiments, we have identified 5 significantly different proteins by 2-DE (two-dimensional electrophoresis) and mass spectrometry. When CGRP affect osteoblast to promote bone healing, these proteins may act as important intermediate neurotransmitters and may be directly or indirectly involved in the metabolism process. More specifically, HRNR Hornerin, a calcium ion binding protein, is very likely the intermediary through which CGRP can affect calcium concentration and regulate cell activity. We will explore this in detail in our later experiments. Further study of CGRP's role in regulating the activation of osteoblasts and osteoclasts during bone wound healing and in promoting growth factors associated with bone trauma will help to more clearly understand the role of neural peptides in this process. We will continue to examine these aspects of osteogenic differentiation in our future research.

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