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Differential Expression of Cytosolic and Nuclear Proteins during S-nitrosoglutathione-induced Cell Death

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Abstract

To examine the involvement of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in nitric oxide-induced apoptosis, subcellular proteomics was performed using cytosolic and nuclear fractions of rat retinal ganglion cells (RGC-5 cells). Proteomic results showed differential distributions of the expression of cytosolic and nuclear proteins following S-nitrosoglutathione (GSNO) treatment. Notably, up-regulation of GAPDH in the nucleus of RGC-5 cells and a concomitant reduction of GAPDH in the cytosolic fraction occurred following GSNO treatment. Moreover, relative GAPDH activity in the cytosol had decreased, while that in the nucleus had increased when subcellular GAPDH activity distribution was examined. Immunocytochemical results also revealed that GAPDH accumulated more densely in the nucleus following GSNO treatment. These subcellular proteomics results confirm that GSNO-induced RGC-5 cell death may occur with translocation and nuclear accumulation of GAPDH.

Keywords: Proteomics, Retinal ganglion cell (RGC-5), S-nitrosoglutathione, Cytosolic and nuclear protein, Glyceraldehyde-3-phosphate dehydrogenase

Introduction

NO (nitric oxide) is a major player in controlling nearly every cellular and organ function in the body. It is an endogenous molecule that functions as a neurotransmitter, a cytoprotective molecule, or a cytotoxic molecule. Certain physiological functions of NO, such as vasodilation and smooth muscle relaxation, are mediated by multiple mechanisms of NO release and NO action[1,2]. Previous studies have shown that the protein Siah1, an E3 ubiquitin ligase, interacts with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the cytosol during cell death in response to induction by NO donors[3-7]. This interaction may play an important role in the translocation of GAPDH from the cytosol to the nucleus[4,5]. The interaction between GAPDH and Siah1 in NO-mediated cell death has been observed in various types of neuronal cells[7].

GAPDH, a classic glycolytic enzyme, catalyzes the reversible conversion of glyceraldehyde-3-phosphate to 1,3-bis-phosphoglycerate in a reaction accompanied by the reduction of NAD^+ to NADH[8]. Long considered to be widely and constitutively expressed, GAPDH is often used as an internal standard for quantitative comparisons. Recently, several studies have revealed that GAPDH is a multifunctional protein and is involved in numerous apoptotic paradigms in diverse cell types, including neuronal and nonneuronal cells[9-11]. GAPDH was first discovered as a pro-apoptotic protein in age-induced cerebellar neuronal apoptosis. Later, the involvement of GAPDH in apoptosis was demonstrated in numerous cell apoptotic paradigms such as aging, cytosine arabinonucleoside and low K^+-induced cerebellar neuron apoptosis[12-14], rat ventral prostate cell apoptosis[15], and tumor necrosis factor-induced thyroid cancer cell apoptosis[16].

We previously reported that GAPDH translocates to and accumulates within the nucleus of retinal ganglion cells (RGC-5 cells) via hyper-pressure, and that its expression increased during hyper-pressure-induced cell death[17]. We also demonstrated that RGC-5 cells differentially express various proteins in response to NO stress. Furthermore, by means of microscopy and immunocytochemistry, S-nitrosoglutathione (GSNO) was shown to stimulate the accumulation and translocation of GAPDH to the nucleus in RGC-5 cells.

Here, we used proteomics analysis to demonstrate the differential expression of various proteins in the cytosolic and nuclear fractions of RGC-5 cells during GSNO-induced cell death. Moreover, increased nuclear localization and concomitant decreased cytosolic expression of GADH was confirmed in GSNO-induced cell death.

Results and Discussion

Inhibition of Cell Growth by GSNO

To examine the effect of GSNO on the growth of

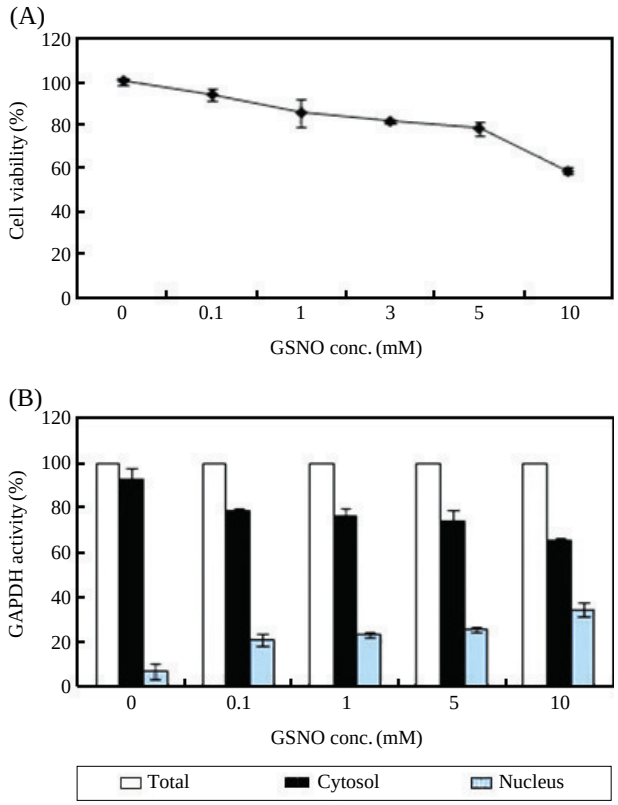


Figure 1. (A) The relative cell viability of RGC-5 cells treated with GSNO for 3 h. RGC-5 cells were challenged with different concentrations of GSNO in DMEM fresh media for 3 h. The cell viability was measured by MTT assay. (B) Subcellular distribution of GAPDH activity. The enzyme activity in the total protein from whole cell before isolating nucleus and cytosol was expressed as 100%.

RGC-5 cells, the cells were incubated in varying concentrations of GSNO. As shown in Figure 1A, GSNO reduced cell viability in a concentration-dependent manner. Exposure to 1 mM and 10 mM GSNO for 3 h resulted in approximately 20% and 40% non-survival of RGC-5 cells, respectively.

Relative Distribution of GAPDH Activity in the Cytosol and Nucleus

To measure the relative distribution of glycolytic activity of GAPDH in RGC-5 cells, GAPDH activity was analyzed after isolating the cytosolic and nuclear fractions from GSNO-treated cells. Note that while the relative enzymatic activities of GAPDH in the cytosol decreased in a GSNO concentration-dependent manner, those in the nucleus increased (Figure 1B). Further statistical analysis demonstrated an approximately 27.6% reduction of GAPDH activity in the cytosolic fraction and concurrent induction of approximately 30 % in the nuclear fraction at 10 mM GSNO. Our observations suggest that GAPDH translocates to the nucleus as a result of GSNO, maintaining in part its dehydrogenase activity, and that this translocation may play a role in programmed cell death.

Differentially Expressed Cytosolic and Nuclear Proteins by GSNO

To confirm whether the level of protein expression of GAPDH in the cytosol might differ from that in the nucleus, cytosolic and nuclear extracts of GSNO-treated cells were collected and protein expression profiles were examined through proteomics. Figure 2A shows 2-D gel electrophoresis (2-DE) images of the cytosolic proteins in RGC-5 cells following 10 mM GSNO treatment for 3 h. More than 296 cytosolic proteins in RGC-5 cells were detected on the 2-DE gels (Figure 2A). To preselect proteins exhibiting variations in expression levels, the proteins from the treated sample and untreated control were compared using ImageMaster 2-DE gel analysis software. After comparing the 2-DE protein patterns on duplicate gels, we found 31 protein spots that were significantly different after GSNO treatment. The protein spots that revealed statistically significant differences were identified by MALDI-TOF MS. The number of matching peptides, the percentage of sequence coverage, and the accuracy of mass estimates were used to evaluate the database search results. In total, 31 proteins were identified in the cytosolic fraction (Table 1). Of those identified, 5 proteins were notably up-regulated and 26 proteins down-regulated. In particular, GAPDH, which plays a key role in apoptosis, decreased markedly. Decreases in stress-induced phosphoprotein 1, peroxiredoxin 2, and transketolase were found, while aldolase 1, ATP-binding cassette protein, and esterase D/formyl glutathione hydrolase increased following GSNO treatment.

When the expression of the nuclear proteins of RGC-5 cells was examined (Figure 2B), 15 protein spots were differentially expressed (Table 2). Heat shock protein 9A, nuclear matrix protein, enolase, and stress 70 protein spots were identified and up-regulated by GSNO in the nucleus. In particular, the expression of GAPDH had increased approximately 5-fold under the GSNO-treated condition versus the untreated control.

Nuclear Accumulation and Translocation of GAPDH

To examine whether GSNO-induced oxidative stress might induce subcellular relocation of GAPDH proteins, the RGC-5 cells were immunostained using anti-GAPDH antibodies. The microscopic immunocytochemical study indicated that immunoparticles were abundantly distributed in the nucleus, whereas they were scarce in the cytoplasm. Furthermore, when cells were

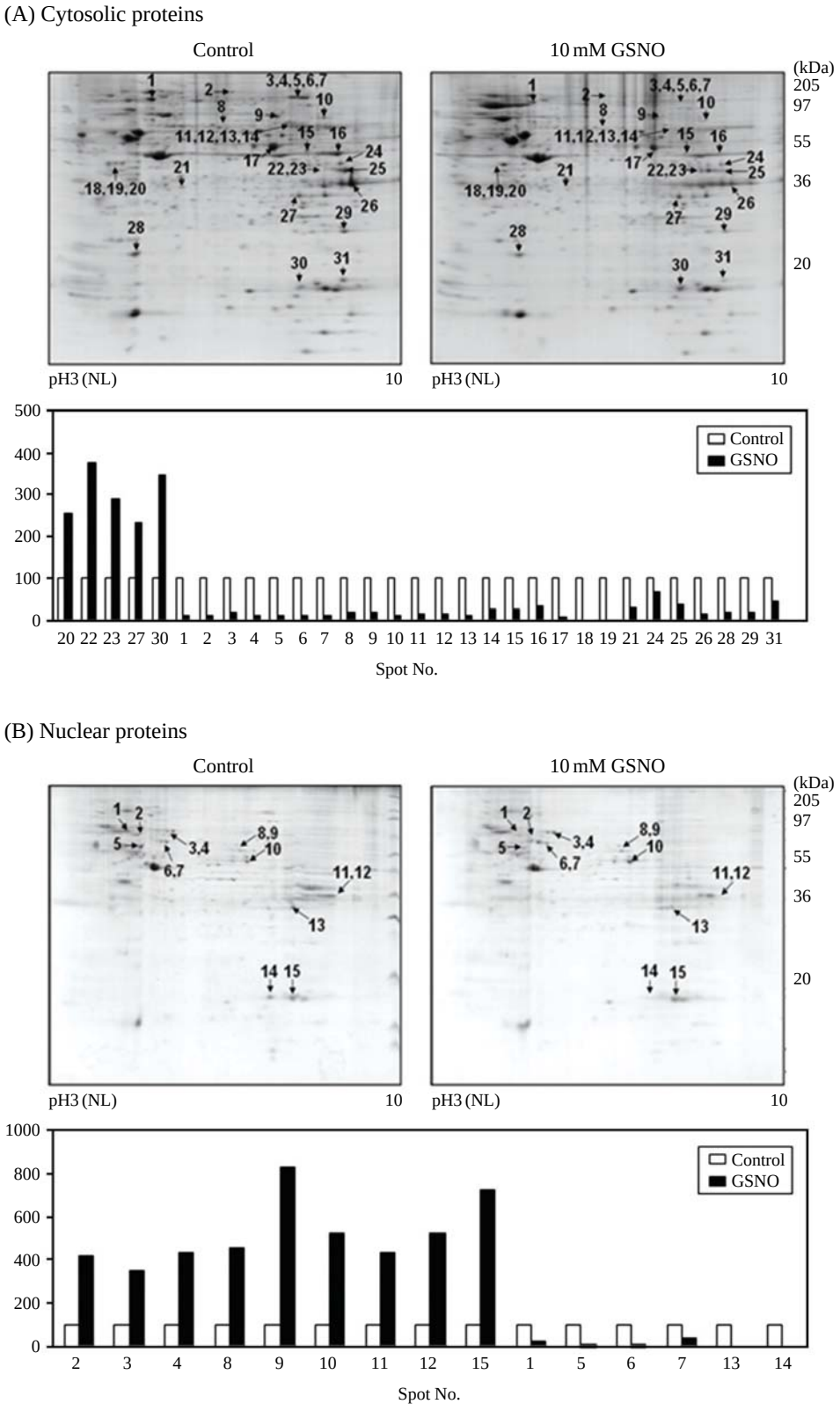


Figure 2. 2-Dimensional gel electrophoretic separation of cytosolic (A) and nuclear proteins (B) isolated from GSNO-treated RGC-5 cells and changes in the protein spots.

Table 1. Differentially expressed cytosolic proteins in rat retinal ganglion cells with S-nitrosoglutathione (GSNO). Arrows indicate up-regulated (↗) and down-regulated (↘) proteins.

Spot No.	Identified protein	Accession No.	Cov %	Matching Peptide No.	Tr/M.W/pI	Change
1	Regulator protein p122-RhoGAP	S54293	9	15	123570/8.1	↘
2	Unnamed protein product	BAC37033	19	28	117300/5.9	↘
3	EeF2 protein	AAH60707	25	19	94510/6.4	↘
4	EeF2 protein	AAH60707	26	19	94510/6.4	↘
5	EeF2 protein	AAH60707	25	17	94510/6.4	↘
6	EeF2 protein	AAH60707	25	18	94510/6.4	↘
7	EeF2 protein	AAH60707	17	13	94510/6.4	↘
8	Unnamed protein product	BAC40527	25	16	57120/5.8	↘
9	Leukotriene A-4 hydrolase	AAB59675	10	10	69500/5.9	↘
10	Transketolase	NP_033414	15	9	68300/7.3	↘
11	Stress induced phosphoprotein 1	NP_058017	27	14	63190/6.4	↘
12	Pyruvate kinase isozyme M2	P52480	25	12	58440/7.2	↘
13	Pyruvate kinase isozyme M2	P52480	28	12	58440/7.2	↘
14	M2-type pyruvate kinase	CCA65761	34	12	58470/7.2	↘
15	Phosphoribosyl aminoimidazole carbolxylase	NP_080215	28	11	47730/7.0	↘
16	Phosphoglycerate kinase 1	P09411	35	8	44920/7.7	↘
17	ADAM 4 protein precursor	AAH85315	10	9	54060/7.7	↘
18	Similar to 40S ribosomal protein SA (p40)	XP484006	21	7	32930/4.6	↘
19	40S ribosomal protein SA (p40)	P38982	29	7	32980/4.6	↘
20	Similar to 40S ribosomal protein SA (p40)	XP_484006	23	7	32930/4.6	↗
21	Annexin A3	NP_038498	24	9	36520/5.3	↘
22	Aldolase 1, A isoform	NP_031464	28	8	39790/8.8	↗
23	Aldolase 1, A isoform	NP_031464	24	7	39790/8.8	↗
24	Unnamed protein product	BAC41097	21	11	40740/7.0	↘
25	Aldolase 1, A isoform	AAH66218	15	8	39750/8.9	↘
26	Glyceraldehyde-3-phosphate dehydrotenase	AAH85315	28	8	36100/7.8	↘
27	Esterase D/formyl glutathione hydrolase	NP_058599	29	9	31870/6.7	↘
28	Peroxiredoxin 2	NP_058865	15	8	21940/5.3	↗
29	Unnamed protein product	BAB27120	36	8	22450/8.6	↘
30	ATP-binding cassette protein	AAF31426	22	11	25100/9.4	↗
31	Cofilin, non muscle	NP_031713	43	8	18770/8.5	↘

treated with higher concentrations of GSNO, more GAPDH protein accumulated in the nucleus (Figure 3). These results suggest the accumulation and translocation of GAPDH to the nucleus with increased GSNO concentrations.

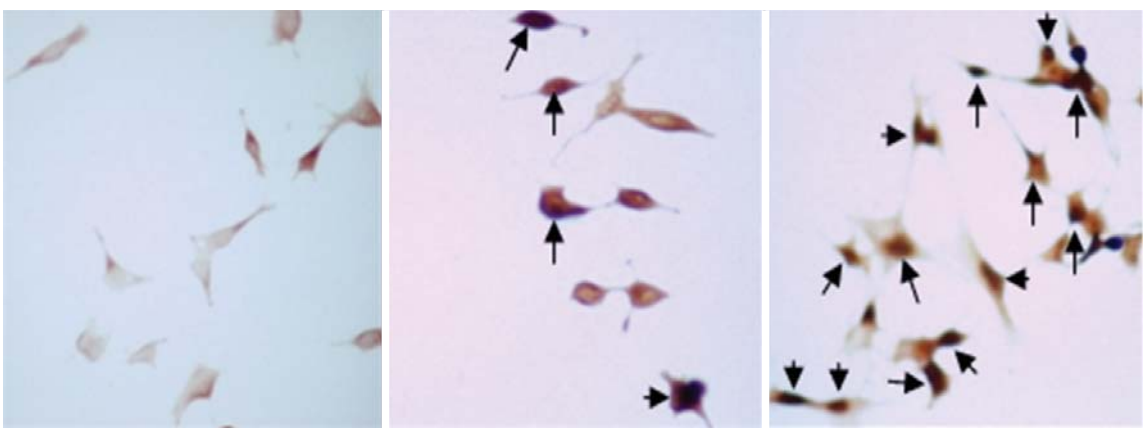
Conclusions

To determine whether cell death induced by NO stress could affect changes in GAPDH, the levels of enzymatic activity and the expression of GAPDH in cytosolic and nuclear extracts were examined in RGC cells following GSNO treatment. When the enzymatic activity of GAPDH in whole lysates, cytosolic, and nuclear fractions were determined, the relative enzymatic activity of GAPDH in cytosolic extracts had decreased, whereas that in nuclear extracts had increased in accordance with the increase in GSNO concentration. We also used a subcellular proteomics approach to identify differentially expressed cytosolic and nuclear proteins in RGC-5 cells after GSNO treatment. Proteomic analysis of cytoplasmic lysates showed that the expression of glucose metabolism-related enzymes including GAPDH, pyruvate kinase, and transketolase was reduced. Moreover, among them, only GAPDH was detected and increasingly expressed in the nucleus. This result was consistent with our previous findings showing the accumulation of GAPDH in the nucleus of RGC-5 cell by hyper-pressure and GSNO through Western blot analysis using whole cell lysates.

The present results show the accumulation of GAPDH in the nucleus and the concomitant reduction of GAPDH in the cytosolic fraction following GSNO treatment. Overall, the findings indicate that the nuclear relocation of GAPDH may be associated with NO-induced apoptosis in retinal ganglion cells. Thus, we suggest that the increase and accumulation of GAPDH in the nucleus may serve as an indicator of GSNO-induced neuronal cell death.

Table 2. Differentially expressed nuclear proteins in retinal ganglion cells with S-nitrosoglutathione (GSNO). Arrows indicate up-regulated (↗) and down-regulated (↘) proteins.

Spot No.	Identified protein	Accession No.	Cov %	Matching Peptide No.	Tr/M.W/pI	Change
1	Hypothetical protein LOC74359	NP_083166	14	9	59450/6.2	↘
2	Unnamed protein product	CAA37653	33	24	59020/5.9	↗
3	Stress 70 protein	O355014	33	26	74000/5.9	↗
4	Heat shock protein 9A	NP_034611	25	23	73800/5.9	↗
5	Vimentin	NP_035811	46	33	53730/5.1	↘
6	Unnamed protein product	CAA37654	32	24	73800/5.9	↗
7	Unnamed protein product	CAA37654	43	31	58080/5.3	↘
8	Unnamed protein product	BAC36493	23	21	57360/6.3	↗
9	Nuclear matrix protein	NP_598890	24	12	55680/6.1	↗
10	Enolase(2-phospho-D-glycerate hydrolyase) (Non neural enolase)	P17182	49	33	47470/6.4	↗
11	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	AAA39509	32	21	36050/9.4	↗
12	Glyceraldehyde-3-phosphatedehydrogenase (GAPDH)	NP_032643	51	31	36050/9.4	↗
13	Guanidine nucleotide binding protein subunit beta 2 like 1	P63245	61	24	35520/7.9	↘
14	Peptidyl-prolyl-cis-trans Isomerase A (PPIase A) (Rotamase A)	P14851	46	14	18050/8.7	↘
15	Unnamed protein product	BAB27089	46	22	18140/8.7	↗



Control 1 mM GSNO 10 mM GSNO

Figure 3. Microscopic analysis showing nuclear translocation of GAPDH in RGC-5 cells treated with 0, 1 and 10 mM GSNO for 3 h. (A) Control (B) 1 mM GSNO (C) 10 mM GSNO.

Materials and Methods

Cell Culture and Reagents

The retinal ganglion cell line, RGC-5[18], was obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT) and maintained at 37°C under an atmosphere of 5% CO_2. A monoclonal antibody for GAPDH was obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and GSNO was obtained from Sigma-Aldrich Co. (St. Louis, MO). A nuclear extraction kit was obtained from Active Motif Co. (Carlsbad, CA).

MTT Assay for Cell Viability

The methyl thiazol tetrazolium (MTT) assay was used to determine cell viability. Briefly, RGC-5 cells were cultured in a 96-well plate (Corning Inc., Corning, NY) at a density of 5×10^3 cells per well. The cells

were then treated with varying concentrations of GSNO (0, 0.1, 1, 3, 5, 10 mM) for 3 h. The cells were then washed and treated with MTT, after which the plates were incubated at 37˚C in the dark for 4 h. After the formation of formazan, 100 µL of dimethyl sulfoxide was added and the absorbance was measured at 570 nm using a microtiter plate reader. The determination of cell viability was then calculated as [(absorbance of the drug-treated sample)/(control absorbance)] × 100[19,20].

Preparation of Whole Cell Lysates and Cytosolic and Nuclear Extracts

RGC-5 cells harvested from T-75 flasks were pelleted by centrifugation (500 × *g*, 10 min). The cell pellets were then resuspended in 20 mM Tris-HCl (pH 7.1) containing 5 mM KCl, 1% aprotinin, and 1 mM $MgCl_2$. Nuclear extracts were prepared using a Nuclear Extract Kit (Active Motif Co.) following the manufacturer's protocol[21].

Enzymatic Assay for GAPDH

The assay mixture (1 mL) contained 10 mM sodium pyrophosphate (pH 8.5), 20 mM sodium phosphate, 0.25 mM NAD^+, and 3 µM dithiothreitol. After incubating in a spectrophotometer at 25˚C for 5 min to achieve temperature equilibrium and establish blank values, the reaction was initiated with the addition of glyceraldehyde-3-phosphate to 0.4 µM. Absorbance at 340 nm was recorded from 0 to 5 min[22,23].

Sample Preparation and Proteomic Analysis

The cytosolic and nuclear extracts of RGC-5 cells were collected by centrifugation (12,000 × *g*, 10 min). The cell pellet was then suspended in sample buffer [0.3% sodium dodecyl sulfate (SDS), 1% 2-mercaptoethanol, 0.05 M TrisHCl (pH 8.0)], and denatured at 100˚C for 3 min. Next, the protein was precipitated by the addition of 10% trichloroacetic acid in acetone at −70˚C for 3 h. For isoelectric focusing (IEF), 500 µg of protein was solubilized in rehydration solution [7 M urea, 2 M thiourea, 4% CHAPS, 100 mM dithiothreitol (DTT), 0.5% ampholyte, bromophenol blue (pH 4-7)]. Immobiline Dry Strips (pH 4-7; Amersham Pharmacia, Piscataway, NJ) were allowed to rehydrate in 400 µL of this protein solution. Immobilized pH gradient (IPG) strips containing 500 µg of protein were then subjected to IEF in a Multiphor II gel apparatus (Amersham Pharmacia) at 20˚C. After IEF, the individual strips were incubated in equilibration solution A [6 M urea, 30% glycerol, 4% SDS, 3.5 mg/mL DTT, 50 mM TrisHCl (pH 6.8)] and then incubated in solution B [6 M urea, 30% glycerol, 4% SDS, 45 mg/mL iodoacetamide, 50 mM TrisHCl (pH 6.8)] for 15 min each. Following equilibration, the proteins were separated by two-dimensional SDS-polyacrylamide gel electrophoresis (2-D SDS-PAGE) using 12.5% gels at 12 W/gel. After electrophoresis, the strips were sealed on the top of the gel using a sealing solution (1% agarose, 0.4% SDS, 0.5 M Tris-HCl). The gels were then run until the bromophenol blue front reached the bottom of the gel, after which they were stained with Coomassie G-250 (17% ammonium sulfate, 3% phosphoric acid, 0.1% Coomassie G-250, 34% methanol). After staining, detection was enhanced by placing the gel in 1% acetic acid. The gels were then destained with 5% acetic acid.

The stained gels were scanned using a UMAX scanner (UMAX Technologies, Plano, TX), and the data were analyzed using the Image Master 2D Elite software (Amersham Pharmacia Biotech, Uppsala, Sweden). Spot detection and matching were performed for each gel, and excised gel spots were analyzed by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) (Micromass, Manchester, UK). To identify the proteins, sequences were searched against the NCBInr and EST databases using the PROFOUND search program and BLAST.

Immunocytochemistry

GAPDH staining was performed using a Histostain-Plus bulk kit (Zymed, San Francisco, CA) according to the manufacturer's instructions. RGC-5 cells were incubated in media containing 1 mM or 10 mM GSNO. After GSNO treatment, the cells were fixed in methanol and 3% H_2O_2 for 10 min. After fixation, the cells were incubated with anti-GAPDH antibody at 4˚C for 1 h and then incubated with biotin-conjugated secondary antibody for 20 min. For visualization, an avidin-peroxidase complex and 3,3-diaminobenzidine (DAB) were used. The stained cells were examined under a microscope and photographed. All steps were followed by three washes with phosphate-buffered saline (PBS). Negative controls were performed by substituting the primary antibody with PBS.

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