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Proteomic characterization of differentially expressed proteins associated with no stress in retinal ganglion cells

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Proteomic analyses of differentially expressed proteins in rat retinal ganglion cells (RGC-5) following S-nitrosoglutathione (GSNO), an NO donor, treatment were conducted. Of the approximately 314 protein spots that were detected, 19 were differentially expressed in response to treatment with GSNO. Of these, 14 proteins were up-regulated and 5 were down- regulated. Notably, an increase in GAPDH expression following GSNO treatment was detected in RGC-5 cells through Western blotting as well as proteomics. The increased GAPDH expression in response to GSNO treatment was accompanied by an increase in Herc6 protein, an E3 ubiquitin ligase. Moreover, GSNO treatment resulted in the translocation of GADPH from the cytosol to the nucleus and its subsequent accumulation. These results suggest that NO stress-induced apoptosis may be associated with the nuclear translocation and accumulation of GAPDH in RGC-5 cells. [BMB reports 2009; 42(7): 456-461]

INTRODUCTION

The glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), is regarded as a housekeeping molecule. As such, GADPH is commonly used as an internal control to standardize gene or protein expression during various molecular techniques. Recent studies have shown that GAPDH is not simply a metabolic enzyme involved in energy production, but a multifunctional protein associated with numerous subcellular processes (1-3). Furthermore, GAPDH has been shown to have several non-glycolytic functions, including roles in neurodegenerative diseases, endocytosis, microtubule bundling, phosphotransferase activity, nuclear RNA export, DNA replication, DNA repair, viral pathogenesis, and apoptosis (4-8). In hypoxic endothelial cells, GAPDH expression has been observed in both the cytosol and the nucleus, and it is believed that the subcellular expression of GAPDH occurs independently of its classical glycolytic function (9). In non-apoptotic cells, GAPDH is primarily found in the extranuclear cytosol, whereas it accumulates within the nuclei of apoptotic cells (10-12). Therefore, its role in apoptosis may be related to its relocalization within the subcellular compartments.

Previous studies have shown that the protein Siah1, an E3 ubiquitin ligase, interacts with GAPDH in the cytosol during cell death in response to induction by NO donors (13-17). This interaction may play an important role in the translocation of GAPDH from the cytosol to the nucleus (14, 15). The interaction between GAPDH and Siah1 in NO-mediated cell death has been observed in various types of neuronal cells (17). As an E3 ubiquitin ligase, Siah1 functions in protein ubiquitination and degradation during apoptosis (18). However, the precise mechanism that triggers the GAPDH-Siah1 interaction and GAPDH translocation, and the function of GAPDH in the nucleus during cell death is currently unknown.

We previously reported that GAPDH translocates to and accumulates within the nucleus of retinal ganglion cells (RGC-5) via hyper-pressure, and that its expression increases during hyper-pressure-induced cell death (19). Here, we used proteomic analysis to demonstrate that RGC-5 cells differentially express various proteins during s-nitrosoglutathione (GSNO)-induced cell death. GAPDH expression in RGC-5 cells increased in response to treatment with GSNO, and this increased GAPDH expression was accompanied by an increase in Herc6 protein, another E3 ubiquitin ligase. GSNO also stimulated the translocation and accumulation of GAPDH within the nucleus in RGC-5 cells.

RESULTS AND DISCUSSION

The growth of retinal ganglion cells was inhibited by GSNO

RGC-5 cells were incubated with various concentrations of GSNO to determine if it affected their growth. After incubation with GSNO for 3 h, the cell viability decreased in a concentration-dependent manner (Data not shown). Specifically, approximately 40% of the RGC-5 cells died after incubation with 10 mM GSNO for 3 h.

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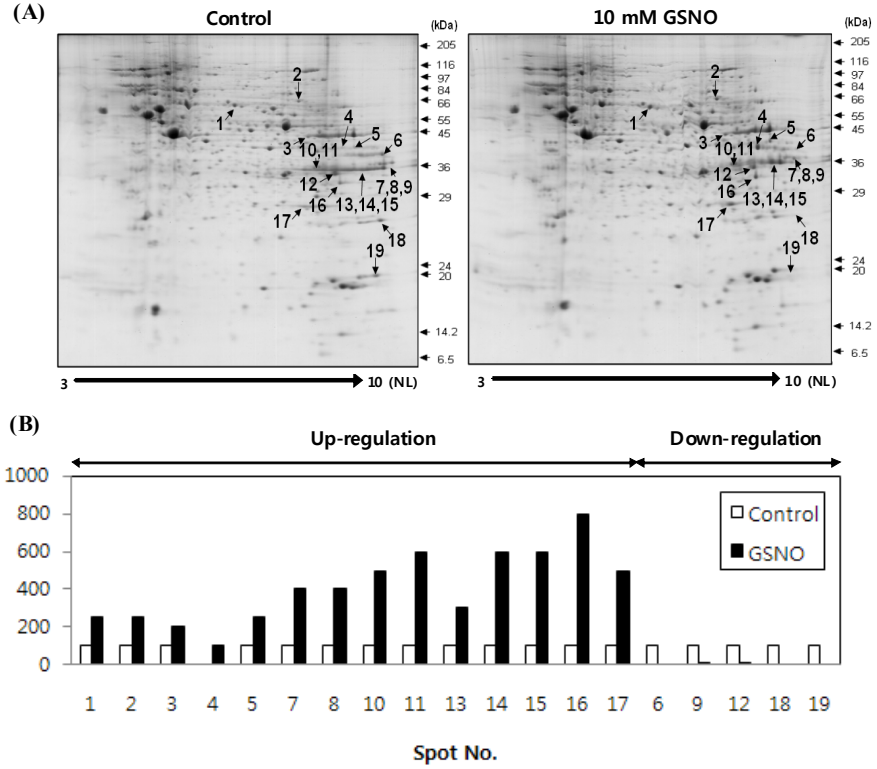

Fig. 1. Differential expression of whole proteins isolated from GSNO-treated RGC-5 cells and changes in the protein spot. Standard 2-D pattern of the whole protein expression (A). The indicated spots (1-19) were differentially expressed in response to 10 mM GSNO. Changes in protein spot 9 (malate dehydrogenase), spot 12 (lactate dehydrogenase A), spot 6 (aldolase 1), spot 2 (stress-induced phosphoprotein 1), spot 11 (aldose reductase), spots 13-15 (GAPDH) and spot 3 (Herc6) were indicated (B).

Proteomic identification of the differentially expressed proteins after GSNO treatment

Protein expression profiles were examined using 2-D SDS-PAGE, followed by MALDI-TOF. After treatment with 10 mM GSNO for 3 h, 314 proteins were detected in the RGC-5 (Fig. 1); however, only 19 proteins that showed changes in abundance of at least 2-fold when compared to the basal level were regarded as up- or down-regulated (Table 1). The expression of metabolism-related proteins, such as malate dehydrogenase, aldolase 1, and lactate dehydrogenase A, decreased, whereas the levels of stress induced phosphoprotein 1 and aldose reductase increased significantly.

Notably, GAPDH expression was enhanced by GSNO. In addition, the E3 ubiquitin ligase subtype, Herc6, also increased in response to treatment with GSNO. Siah1, another type of E3- ubiquitin ligase, has been implicated in NO/GAPDH/Siah1 death signaling in NO stress-induced cell death (13-15). It has also been reported that GAPDH may be a major target of NO in cell death based on observation of the NO-induced ADP ribosylation or other modification of GAPDH (20). In addition, NO-nitrosylation has been found to enable GAPDH to bind Siah1, an E3-ubiquitin ligase. This binding results in the formation of a GAPDH/Siah1 complex, which has been found to be associated with the nuclear localization of GAPDH (13-16). The results of the present study suggest that GAPDH may be complexed with Herc6 in NO stress-induced cell death in RGC-5 cell as a death signal.

Western blot of GAPDH

We investigated GAPDH to determine if it is involved in NO stress-induced cell death by examining its expression following GSNO treatment (Fig. 2). Western blot analysis revealed that the overall expression of GAPDH in whole cell lysate increased in response to treatment with increasing GSNO concentrations. Interestingly, GSNO treatment caused a concentration-dependent decrease in the cytosolic expression of GAPDH, whereas its nuclear expression increased significantly. In a previous study, hyper-pressure-induced apoptosis resulted in aberrant GAPDH protein expression and the nuclear accumulation of GAPDH in retinal ganglion cells (19).

The nuclear localization of GAPDH was recently reported to participate in the cell death cascade (21-23). Other studies have revealed that GAPDH is functionally diverse as both a cytosolic and a nuclear protein, and that these activities occur independently of its classical glycolytic function (24, 25).

Proteomic and Western blotting analyses demonstrated that GAPDH protein accumulates in the nucleus during NO stress-induced cell death. In addition, immunocytochemistry revealed that abundant immunoreactive particles were distributed in the nucleus, whereas immunoreactivity in the cytoplasm was sparse. The majority of GAPDH is presumed to localize in the cytoplasm, where glycolysis occurs. However, we

Table 1. Differentially expressed 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	Prolactin-like protein-F beta	NP_001008342	16	13	29030/5.4	↗
2	Stress induced phosphoprotein 1	NP_058017	36	18	63190/6.4	↗
3	Herc6 protein	AAH85921	8	11	117730/8.7	↗
4	Cathepsin Y	NP_899159	23	10	34860/6.8	↗
5	Unnamed protein product	BAC41097	31	13	40740/7.0	↗
6	Aldolase 1, A isoform	NP_031464	32	9	39790/8.8	↘
7	Heterogeneous nuclear ribonucleoprotein A2/B1	AAK98601	37	7	36020/8.7	↗
8	Heterogeneous nuclear ribonucleoprotein A2/B1	AAK98601	46	10	36020/8.7	↗
9	Malate dehydrogenase	AAA39509	31	9	36050/9.4	↘
10	Annexin A1 (Annexin 1) (Calpactin Ⅱ) (Chromobidin-9) (p35) (Phospholipase A2 inhibitory protein)	P10107	34	11	39000/7.0	↗
11	Aldose reductase (AR)	P45376	33	9	36060/6.7	↗
12	Lactate dehydrogenase 1, A chain	NP_034829	36	14	36820/7.8	↘
13	Glyceraldehyde-3-phosphate dehydrogenase	P04797	32	9	36100/8.7	↗
14	Glyceraldehyde-3-phosphate dehydrogenase	P04797	25	6	36100/8.7	↗
15	Glyceraldehyde-3-phosphate dehydrogenase	P04797	28	8	36100/8.7	↗
16	Guanine-nucleotide-binding protein subunit beta 2 like 1	P63245	43	16	35520/7.9	↗
17	Triosephosphate isomerase 2	NP_033441	50	12	27040/6.9	↗
18	Immunoglobulin heavy chain variable region	AAF66932	34	8	11230/9.0	↘
19	Immunoglobulin heavy chain variable region	CAB71389	47	7	12120/9.3	↘

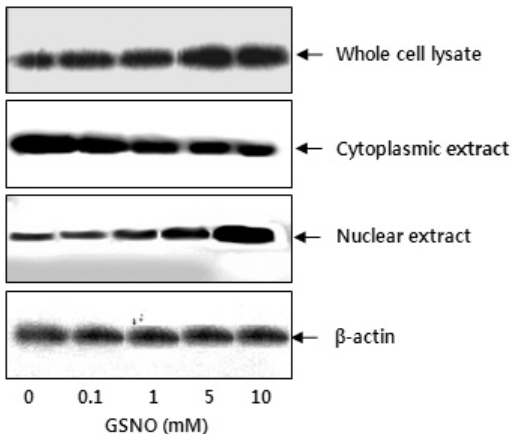

Fig. 2. RGC-5 cells were incubated with GSNO for 3 h and then subjected to Western blot analysis to analyze the GAPDH expression.

found that increasing amounts of GAPDH accumulated in the nucleus in response to treatment with increasing concentrations of GSNO. These findings suggest that glycolytic activity is not an essential function in the nucleus, and that nuclear GAPDH has an alternate function.

Subcellular relocalization of GAPDH

To determine if NO stress induces the relocalization of GAPDH in RGC-5 cells, we used immunofluorescent staining to examine the subcellular localization of GAPDH. After incubation with GSNO for 3 h, GAPDH accumulated in the nucleus of RGC-5 cells, whereas it localized to the cytosol in control cells (Fig. 3). Moreover, this nuclear accumulation of GAPDH occurred in a concentration-dependent manner, presumably via the translocation of GAPDH from the cytosol.

Our results suggest that the translocation of GAPDH from the cytosol to the nucleus may be functionally related to GSNO-induced cell death. In addition, the aberrant expression of GAPDH protein in the nucleus may play a unique role in GSNO-induced cell death. Furthermore, the increased expression and nuclear accumulation of GAPDH may serve as an indicator of NO stress. Overall, the results of this study indicate that the nuclear relocalization of GAPDH may be closely associated with NO-induced apoptosis in retinal ganglion cells.

MATERIALS AND METHODS

Cell culture and reagents

The retinal ganglion cell line, RGC-5 (26), 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) and maintained at 37°C under an atmosphere of 5% CO_2. Monoclonal antibody for GAPDH was obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and GSNO was obtained from

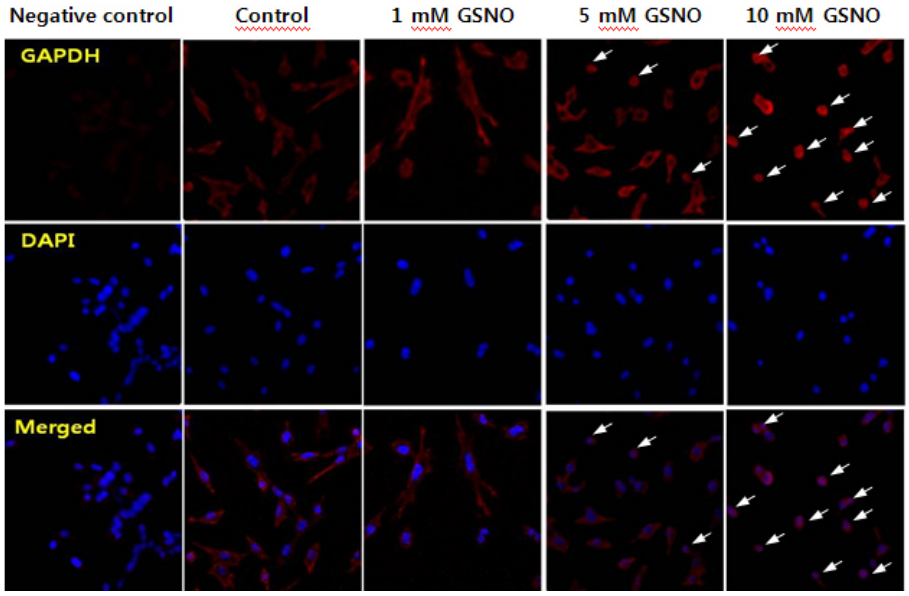

Fig. 3. Nuclear translocation of GAPDH in RGC-5 cells treated with 0, 1, 5 and 10 mM GSNO for 3 h. Nuclei were revealed by counterstaining with DAPI, while GAPDH was detected by anti-GAPDH monoclonal antibody and Texas-red-conjugated anti-horse-IgG.

Sigma-Aldrich Co. (St. Louis, MO). A nuclear extraction kit was obtained from Active Motif Co. (Carlsbad, CA).

MTT assay for cell viability

The 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 and 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 DMSO were 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 (27).

Sample preparation and two-dimensional gel electrophoresis

RGC-5 cells were collected by centrifugation at 12,000 × g for 10 min. The cell pellet was then suspended in sample buffer (0.3% SDS, 1% 2-mercaptoethanol, 0.05 M Tris-HCl, pH 8.0), and denatured at 100°C for 3 min. Next, the protein was precipitated by the addition of 10% TCA in acetone at -70°C for 3 h. For isoelectric focusing (IEF), 500 µg of protein were solubilized in rehydration solution (7 M urea, 2 M thiourea, 4% CHAPS, 100 mM 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. 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 Tris-HCl, pH 6.8), and then incubated in solution B (6 M urea, 30% glycerol, 4% SDS, 45 mg/ml iodoacetamide, 50 mM Tris-HCl, 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 following voltage/time profile was used: linear increase from 0 to 500 V at 1,000 V/h, 500 V at 2,000 V/h, linear increase from 500 to 3,500 V at 10,000 V/h, and a final phase of 3,500 V at 35,000 V/h, up to a maximum of 48,000 V/h (28).

Mass spectrometry analysis

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 the excised gel spots were analyzed by MALDI-TOF (matrix-assisted laser desorption/ionization-time of flight) mass spectrometry (Micromass, Manchester, UK). Tryptic peptides derived from protein spots were analyzed and amino acid sequences were deduced using the de novo peptide-sequencing program, PepSeq (Gibbsland). To identify the proteins, sequences were searched against the NCBInr and EST databases using the PROFOUND search program (http://www.rockefeller.edu/labheads/chait/novel_tandem.php)

and BLAST.

Confocal micrpscopic analysis for the detection of GAPDH

RGC-5 cells (5×10^4 cell/well) were seeded on a poly-L-lysine coated cover-slip in a 12-well culture dish. Following treatment with the nitrosative stressor, GSNO, at 0-10 mM for 3 h, the cells were washed with Ca^{2+}- and Mg^{2+}-PBS, and then fixed with 3.7% paraformaldehyde for 10 min. Next, the cells were washed with Ca^{2+}- and Mg^{2+}-PBS, and then incubated with PBS containing 0.1% Triton X-100 (PBST) for 10 min. After washing, the cells were blocked with blocking solution (10% normal horse serum, 0.1% BSA in Ca^{2+}- and Mg^{2+}-PBS) for 1 h. The cells were then incubated with a 1 : 200 dilution of anti-GAPDH monoclonal antibody overnight at 4°C. After washing, the coverslips were incubated with a 1 : 400 dilution of Texas-red-conjugated anti-horse IgG (Vector Laboratories, Burlingame, CA) in the dark for 1 h at room temperature. Between each step, the cells were washed with Ca^{2+}- and Mg^{2+}-PBS. The nuclei were then stained with 0.5 μg/ml DAPI (4,6-diamidino-2-phenylindole). The samples were then washed twice with PBS, after which the coverslips were mounted with mounting medium (Vector Laboratories) and observed with the aid of a confocal microscope (Carl Zeiss).

Preparation of whole cell lysates and nuclear extracts

RGC-5 cells harvested from T 75 flasks were pelleted by centrifugation at 500 × g for 10 min. The cell pellets were then resuspended in 20 mM Tris-HCl (pH 7.1) containing 5 mM KCl, 1% aprotitnin and 1 mM $MgCl_2$. Nuclear extracts were obtained using a Nuclear Extract Kit (Active Motif, CA) following the manufacturer's protocols.

Western blot analysis

After GSNO treatment, RGC-5 cells were lysed in ice-cold extraction buffer (20 mM Tris-HCl, 1 mM EDTA, 0.5 mM PMSF, 1% NP-40, pH 7.5) for 30 min at 4°C, and then centrifuged at 12,000 × g for 15 min. The total cellular proteins were then denatured and resolved by 12% SDS-PAGE. Next, the membrane was transferred to a polyvinylidene difluoride (PVDF) membrane and then saturated with PBS-Tween 20 and 5% milk. The membrane was then incubated overnight with the anti-GAPDH monoclonal antibody at 4°C, followed by incubation with horseradish peroxidase-conjugated anti-mouse IgG at room temperature for 1 h. The membranes were then visualized using the ECL plus Western-blotting detection system.

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