

## p53 is not necessary for nuclear translocation of GAPDH during NO-induced apoptosis

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**Aberrant GAPDH expression following S-nitrosoglutathione (GSNO) treatment was compared in HepG2 cells, which express functional p53, and Hep3B cells, which lack functional p53. The results of Western blotting and fluorescent immunocytochemistry revealed that nuclear translocation and accumulation of GAPDH occur in both HepG2 and Hep3B cells. This finding suggests that p53 may not be necessary for the GSNO-induced translocation of GAPDH to the nucleus during apoptotic cell death in hepatoma cells. [BMB reports 2011; 44(12): 782-786]**

### INTRODUCTION

Nitric oxide (NO) is an important biological messenger that has multiple effects in pathophysiological conditions such as inflammation, endothelium-dependent vasorelaxation, and septic shock (1, 2). Some of its effects are based on its redox chemistry. Notably, the interaction of NO with sulfhydryl groups was reported to affect the cellular functions of proteins (3). Previous studies have demonstrated that NO can trigger lethal reactions including apoptotic cell death in different systems (4).

Expression of wild type p53, a tumor suppressor gene, is known to be closely linked to apoptotic cell death (5, 6). Moreover, NO-induced apoptosis is thought to be triggered by the subsequent expression of p53 (3). One of the most abundant endogenous nitrosothiols, S-nitrosoglutathione (GSNO), exhibits NO-like biological activities, and the release of NO from GSNO promotes NO-induced apoptosis (7). Moreover, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) translocates to the nucleus during apoptotic cell death caused by several environmental stressors, including hyper-pressure, as we reported previously (8). We recently showed that GSNO-induced apoptosis in RGC-5 cells is accompanied by nuclear translocation and the accumulation of GAPDH (9, 10).

It is evident that the glycolytic enzyme GAPDH is not simply a metabolic protein involved in energy production, but is a multifunctional protein involved in numerous subcellular processes (11-13), including endocytosis, microtubule bundling, phosphotransferase activity, nuclear RNA export, DNA replication, DNA repair, viral pathogenesis, oncogenesis, and apoptosis (14, 15). The role of GAPDH in apoptosis has been described in the context of neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, and Huntington's disease (16-18). Its role in apoptosis in various neuronal cell types has been shown to be related to its subcellular relocalization. However, the mechanisms underlying the translocation of GAPDH and its function in the nucleus remain unclear (8, 19-22). A recent study showed that the nuclear translocation of GAPDH during cell death requires S-nitrosylation, which triggers binding to the E3 ubiquitin ligase Siah-1, a potential carrier/shuttle protein (7, 22, 23). Loss of wild type p53 activity has traditionally been associated with resistance to apoptosis and malignant transformation. However, the absence of p53 rendered vascular smooth muscle cells more susceptible to NO-induced apoptosis (23). Moreover, p53-negative U937 cells exhibited p53-independent signaling during NO-mediated apoptosis (24).

Based on evidence that suggests apoptotic cell death and the nuclear translocation of GAPDH are intimately linked, and given the importance of p53 to the nuclear translocation of GAPDH, we conducted studies to determine the contribution of p53 to GAPDH translocation during NO-induced apoptosis. Surprisingly, we found that translocation of GAPDH to the nucleus occurred in both Hep3B cells, which lack functional p53, and HepG2 cells, which express functional p53. We therefore suggest that the nuclear relocalization of GAPDH during NO-induced apoptotic cell death in hepatoma cells does not depend on the presence of p53.

### RESULTS AND DISCUSSION

#### Inhibition of cell viability by GSNO

Reactive nitrogen species (RNS) have recently been shown to regulate cell growth, survival, and apoptosis in a variety of cell types (25). RNS such as GSNO are thought to function as intracellular signaling molecules during oxidative stress and the induction of apoptotic cell death. To test whether nitrosative stress induced by GSNO affects the viability of two hepatoma cell lines, HepG2 and Hep3B, cells were exposed to 0-12 mM GSNO. As shown in

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<http://dx.doi.org/10.5483/BMBRep.2011.44.12.782>

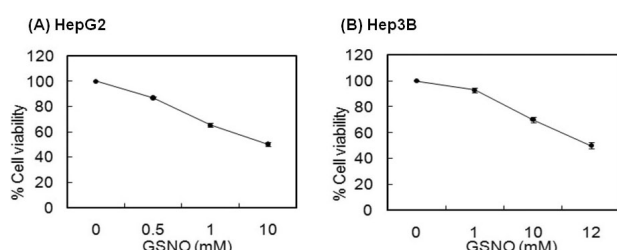
Received 9 August 2011, Accepted 16 September 2011

**Keywords:** Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Hepatoma cells, Nuclear translocation, p53, S-nitrosoglutathione (GSNO)

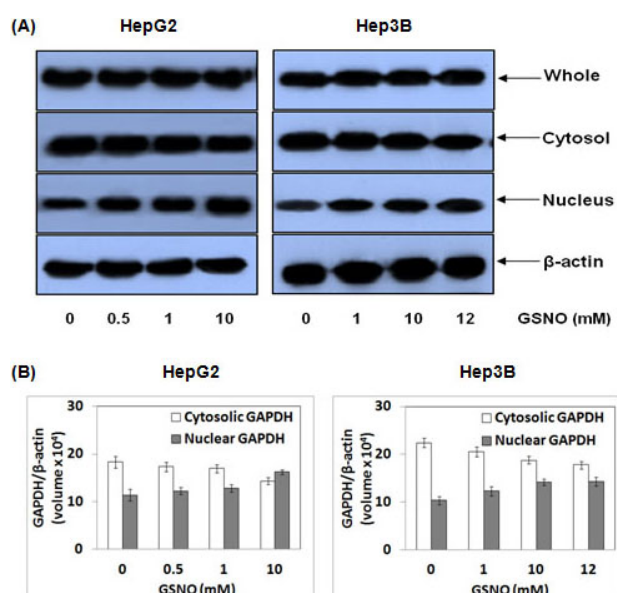
Fig. 1, GSNO inhibited cell viability in a dose-dependent manner. The proliferation of HepG2 and Hep3B cells were approximately 50% inhibited at 10 and 12 mM GSNO, respectively.

### Western blot analysis of GAPDH

Western blot analysis revealed that the overall expression of GAPDH in HepG2 and Hep3B whole-cell lysates was unaffected by increasing concentrations of GSNO. As shown in Fig. 2, GSNO treatment caused a concentration-dependent decrease in the cytosolic expression of GAPDH, whereas its nuclear expression increased significantly in HepG2 cells. Interestingly, increasing concentrations of GSNO also caused a notable increase



**Fig. 1.** Relative viability of HepG2 and Hep3B cells after GSNO treatment, as assessed by MTT assay. HepG2 (A) and Hep3B (B) cells were challenged with different concentrations of GSNO in fresh DMEM medium for 3 h.



**Fig. 2.** (A) Western blot analysis of GAPDH expression in whole-cell, cytosolic, and nuclear extracts of GSNO-treated HepG2 and Hep3B cells. HepG2 and Hep3B cells were incubated with GSNO for 3 h and then subjected to Western blot analysis of GAPDH expression. (B) Nuclear and cytosolic GAPDH levels were normalized to β-actin, and band sizes in the Western blots were analyzed using ImageQuant TL software (GE Healthcare Biosciences).

in the nuclear expression of GAPDH in Hep3B cells, while cytosolic GAPDH levels decreased. This result raises the possibility that the nuclear translocation of GAPDH during GSNO-induced apoptotic cell death in hepatoma cells does not require the presence of p53.

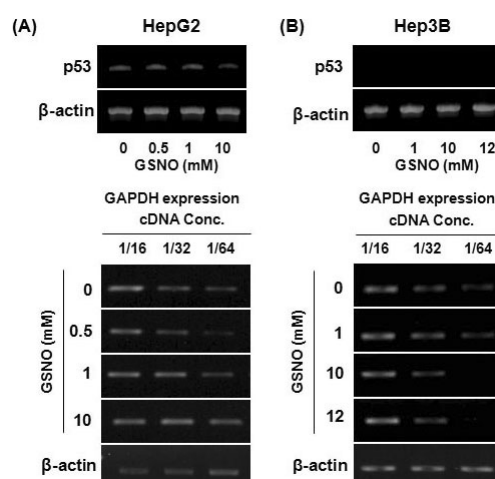
### Semi-quantitative RT-PCR analysis of GAPDH expression

Human HepG2 hepatoma cells, which express functional p53, and Hep3B cells, which lack functional p53, have frequently been used in the evaluation of p53 dependency (26). In order to determine whether p53 is involved in the aberrant translocation of GAPDH during NO stress-induced apoptosis, GAPDH gene expression was compared in p53-positive HepG2 cells and p53-negative Hep3B cells.

As shown in Fig. 3A and 3B, we confirmed the presence of p53 transcripts in HepG2 cells, and their absence in Hep3B cells. Overall GAPDH mRNA expression decreased as the GAPDH cDNA was serially diluted. GAPDH transcripts were not expressed in Hep3B cells treated with 10 mM GSNO (which show about 70% cell viability), but were detected in cells treated with 1 mM GSNO (90% cell viability), even when 1/64-diluted cDNA was used. On the other hand, GAPDH transcripts were strongly expressed in HepG2 cells treated with 10 mM GSNO (which showed 50% cell viability). These results suggest that GAPDH mRNA expression during GSNO-induced cell death is lower in Hep3B cells than in HepG2 cells.

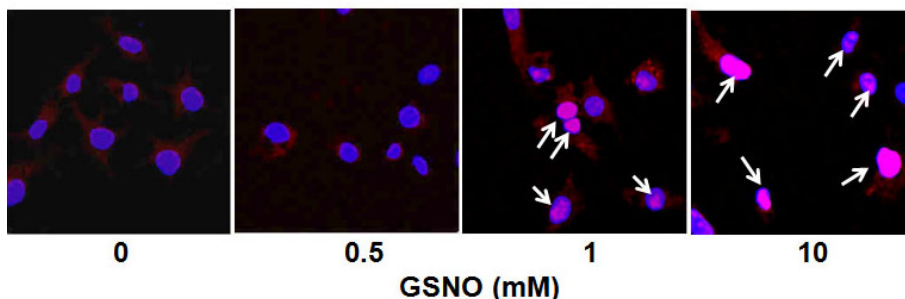
### Subcellular relocalization of GAPDH

The nuclear relocalization of GAPDH was recently reported as part of the cell death cascade (8, 16–18). To investigate the relocalization of GAPDH in HepG2 and Hep3B cells, the subcellular localization of GAPDH was compared by fluorescent immunostaining using specific antibodies against GAPDH. As shown in

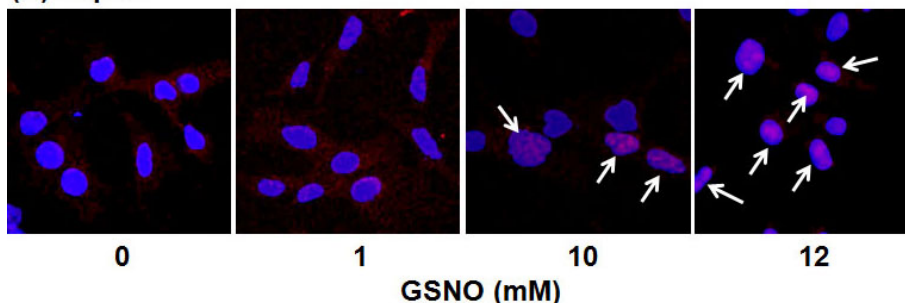


**Fig. 3.** Semi-quantitative RT-PCR analysis of GAPDH gene expression in GSNO-treated p53-positive HepG2 cells (A) and p53-negative Hep3B cells (B).

### (A) HepG2



### (B) Hep3B



**Fig. 4.** Subcellular localization of GAPDH in GSNO-treated HepG2 cells (A) and Hep3B cells (B). The cells were evaluated by confocal microscopy. Red fluorescence, GAPDH; blue fluorescence, DAPI (nuclei). Arrows show nuclear GAPDH staining.

Fig. 4A, abundant fluorescent immunoreactive particles were distributed in the nucleus following the treatment of HepG2 cells with GSNO, whereas immunoreactivity in the cytoplasm was limited, as expected. Interestingly, GAPDH also accumulated in the nucleus of GSNO-treated Hep3B cells, although the level was lower than that in HepG2 cells (Fig. 4B). These results show the unequivocal nuclear translocation and accumulation of GAPDH during GSNO-induced cell death in both HepG2 and Hep3B cells. The presence of p53 is not necessary for the translocation of GAPDH to the nucleus during GSNO-induced apoptotic cell death.

In this study, we demonstrate that the NO donor GSNO triggers the nuclear translocation and accumulation of GAPDH in both p53-negative Hep3B cells and p53-positive HepG2 cells. Therefore, the nuclear translocation of GAPDH may be associated with p53-independent signaling during apoptotic cell death in Hep3B cells.

## MATERIALS AND METHODS

### Cell culture and reagents

HepG2 and Hep3B human hepatocellular carcinoma cell lines were obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in Dulbecco's modified Eagle medium (DMEM, HyClone) supplemented with 10% heat-inactivated fetal bovine serum (FBS, HyClone) and 1% penicillin-streptomycin (HyClone), and maintained at 37°C in a 5%-CO<sub>2</sub> atmosphere. A monoclonal antibody-specific GAPDH was obtained

from Abcam (MA), and GSNO was obtained from Sigma-Aldrich Co. (St. Louis, MO). A nuclear extract kit was obtained from Active Motif (Carlsbad, CA).

### MTT cell viability assay

An MTT assay was used to assess cell viability. Briefly, HepG2 and Hep3B cells were cultured in 96-well plates (Corning Inc., Corning, NY) at a density of  $5 \times 10^3$  cells per well. The cells were then treated with varying concentrations of GSNO (0, 0.1, 0.5, 1, 10, and 12 mM) for 3 h. The cells were washed and treated with MTT, after which the plates were incubated at 37°C in the dark for 2 h. After the formation of formazan, 100 µl of DMSO was added and the absorbances were measured at 570 nm using a microtiter plate reader. Cell viability was then calculated as [(absorbance of the GSNO-treated sample) / (absorbance of the control sample)]  $\times$  100 (27). Each experiment was repeated three times with triplicate samples per treatment. Error bars represent standard error of the mean (SEM).

### Semi-quantitative RT-PCR

Total RNA was obtained from each cell line according to the manufacturer's instructions. To prepare the template for the reverse transcription polymerase chain reaction (RT-PCR), first-strand complementary DNA (cDNA) was synthesized from individual total RNA samples isolated from human hepatoma cell lines in a reaction primed with a gene-specific primer. The primer sequences are as follows: p53 forward, 5'-TCCCCTGC GCTCAACAAGAT-3'; p53 reverse, 5'-TCGTGGTGAGGCTC

CCCTT-3'; GAPDH forward, 5'-GTCAAGGCTGAGAACGGG AAG-3'; GAPDH reverse, 5'-CATCACGCCACAGTTTCCCG-3';  $\beta$ -actin forward, 5'-TATGCCCTCCCCATGCCAT-3'; and  $\beta$ -actin reverse, 5'-AGAAGCATTTGCGGTGGACGAT-3'. The cDNA was serially diluted (1/16, 1/32, and 1/64) for RT-PCR, which was performed by priming with antisense primers for p53, GAPDH, and  $\beta$ -actin.  $\beta$ -actin was chosen as an endogenous internal control to which the levels of the other PCR amplification products were normalized.

### Preparation of whole-cell lysates and nuclear extracts

After GSNO treatment, HepG2 and Hep3B cells were lysed through incubation 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. Nuclear extracts were obtained using a nuclear extract kit (Active Motif, CA) according to the manufacturer's protocols.

### Western blot analysis

Protein samples were denatured and resolved by 10% SDS-PAGE. Next, the separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane and then saturated with PBS-Tween 20 and 5% milk. The membrane was then incubated overnight at 4°C with anti-GAPDH monoclonal antibody, and with horseradish peroxidase-conjugated goat anti-mouse IgG for 1 h at room temperature. After washing three times with PBST, the membrane was finally visualized using ECL Plus Western blotting detection reagent (Amersham Pharmacia Biotech, UK) (28).

### Confocal microscopic analysis of GAPDH

HepG2 and Hep3B cells were seeded to a poly-L-lysine-coated cover-slip in a 12-well culture dish. They were then treated with the nitrosative stressor GSNO (0-12 mM) for 3 h. The cells were washed with  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -PBS, and then fixed through incubation with 3.7% paraformaldehyde for 10 min. Next, the cells were washed 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  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -PBS) for 1 h. The cells were then incubated overnight at 4°C with anti-GAPDH antibody (dilution 1:200). After washing, the cells were incubated in the dark for 1 h at room temperature with Texas Red-conjugated anti-horse IgG (Vector Laboratories, Burlingame, CA) (1:400). Between steps, the cells were washed with  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -PBS. The nuclei were then stained with 0.5  $\mu\text{g}/\text{ml}$  DAPI (4,6-diamidino-2-phenylindole). The samples were washed twice with  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -PBS, mounted on cover slips using mounting medium (Vector Laboratories), and visualized using a confocal microscope (Carl Zeiss).

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