Mitochondria Are an Essential Mediator of Nitric Oxide/Cyclic Guanosine 3′,5′-Monophosphate Blocking of Glucose Depletion–Induced Cytotoxicity in Human HepG2 Cells

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Abstract
It is well known that glucose is a major energy source in tumors and that mitochondria are specialized organelles required for energy metabolism. Previous studies have revealed that nitric oxide (NO) protects against glucose depletion–induced cytotoxicity in mouse liver cells and in rat hepatocytes, but the detailed mechanism is not well understood. Therefore, we investigated the involvement of mitochondria in the NO protective effect in human hepatoma HepG2 cells. In this study, we showed that glucose depletion resulted in a time-dependent decrease in intracellular NO and in the protein expression of NO synthases. This glucose depletion–induced decrease in NO was blocked by NO donors. Next, we showed that the cytoprotective effect of NO is via a cyclic guanosine 3′,5′-monophosphate–dependent pathway. Additionally, SNP blocked a glucose depletion–induced decrease in mitochondrial mass, mitochondrial DNA copies, and ATP level in HepG2 cells. Moreover, glucose depletion decreased the expression of various mitochondrial proteins, including cytochrome c, complex I (NADH dehydrogenase), complex III (cytochrome c reductase), and heat shock protein 60; these glucose depletion–induced effects were blocked by SNP. Furthermore, we found that rotenone and antimycin A (mitochondria complex I and III inhibitors, respectively) blocked SNP cytoprotection against glucose depletion–induced cytotoxicity. Taken together, our results indicated that the mitochondria serve as an important cellular mediator of NO during protection against glucose deprivation–induced damage.

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Introduction
Hepatocellular carcinoma is the fifth most frequent malignancy in the world and the leading cause of tumor-related death in Taiwan (1). Prognosis for hepatocellular carcinoma is poor mainly because there is a lack of an effective treatment. Hepatocellular carcinoma is resistant to chemotherapy and therefore multidrug resistance remains a significant impediment to successful chemotherapy. On the other hand, radiation therapy has serious side effects because normal liver tissue is also radiation sensitive. Current conformal radiotherapy techniques permit treatment of the target volume with a high radiation dose while minimizing damage to normal tissue. Although surgery is effective in the treatment of patients with small hepatocellular carcinoma, the number of patients suitable for surgical therapy is limited. Rapid tumor growth and metastasis have been associated with poor survival (2). As a result of the above, new preventive and therapeutic strategies are focusing on targets within the molecular pathways leading to hepatocellular carcinoma (3).

Cancer cells prefer glycolysis in the presence or absence of oxygen and this is well known as the Warburg effect (4). Alterations in the tumor microenvironment, which is characterized by regions of fluctuating and chronic hypoxia, low extracellular pH, and low glucose concentration contribute significantly to tumor progression (5). In a nutrient-insufficient environment, cancer cells can survive and continue to grow by activation of survival pathways. Fast-growing tumor cells show an increase in glycolysis caused by up-regulation of glucose transporters and hexokinase activity (6). In addition, oncogenes such as Ras has been found to induce increased glycolysis and to decrease dependence on the mitochondria energy supply in human primary fibroblast cells during malignant transformation (7). HepG2 hepatoma cells have been found to have increased glycolytic enzymes (8) and is a model system frequently used to study the effects of glucose depletion (9–11). Tumor cells adjust to the use of glycolytic mechanisms to provide a supply of ATP rather than using oxidative phosphorylation, and this suggests that glucose is an important energy source for cancer cells.

Mitochondria supply cell energy by synthesis of ATP through oxidative phosphorylation. It is an energy-producing organelle and it plays an important role in sensing cellular energy requirement. Mitochondrial dysfunction is frequently found in tumors (12). We have previously reported that mitochondrial DNA (mtDNA) copy number decreases along
with an impairment of mitochondrial biogenesis in hepatoma tissue (13). In addition, somatic mtDNA mutations are also found in breast cancer tissue (14). Alterations in mitochondria, including mtDNA deletion, point mutations, duplications, and insertions, have been reported in a variety of cancers (15). Environmental changes, such as variation in nutrient supply, have been found to regulate gene expression. Glucose depletion is a metabolic stressor that triggers several signal transduction pathways. Glucose depletion results in a cellular stress-induced endoplasmic reticulum response, which includes an increased expression of heme oxygenase-1 gene and of glucose-regulated protein 78 (16). In addition, it has been reported that glucose depletion results in a decrease in the mitochondrial membrane potential, which leads to mitochondrial dysfunction and death in PC12 cancer cells (17). Moreover, inhibition of energy metabolism in hepatoma cells causes cell death by depletion of mtDNA, suppression of glucose transporter expression, and lowering of glucose uptake (18). These results together suggest that mitochondria play a central role when responding to variation in energy supply and cell survival.

Nitric oxide (NO), a small signaling molecule produced by NO synthase (NOS), is involved in biological functions such as blood vessel dilation and the induction of the inflammatory response. It has been found that modulation of energy production can either lead to NO-induced cytotoxicity or cytoprotection under different conditions. For example, NO-induced cytotoxicity in phaeochromocytoma cells is mediated by energy depletion via inhibition of mitochondrial respiration (19). NO-induced cell apoptosis or necrosis depends on the glucose concentration in neuronal cells (20). In addition, NO has also been found to protect murine embryonic liver cells from glucose depletion–induced cytotoxicity by up-regulation of heme oxygenase-1 expression (21). NO reversibly competes with oxygen binding to cytochrome c oxidase in the mitochondrial respiratory chain. However, recent studies showed that NO can also increase mitochondrial biogenesis and the ATP level in mammalian cells and tissues (22, 23). They showed that NO triggered mitochondrial biogenesis through activation of guanylate cyclase and generation of cyclic guanosine 3′,5′-monophosphate (cGMP). Therefore, NO-induced effects are closely related to mitochondrial function in cancer cells.

As far as we know, the effect of NO on mitochondria during glucose depletion in hepatoma cells is not clear, and there is only limited information available on whether mitochondria are involved in NO effect on glucose depletion–induced cell damage. In this study, we found that glucose depletion decreased intracellular NO and the presence of a NO donor protected against glucose depletion–induced cytotoxicity by modulation of not only mitochondrial biogenesis but also mitochondrial function in hepatoma cells.

**Results**

*The Effect of Glucose Depletion and Sodium Nitroprusside on the Endogenous Level of NO in Human Hepatoma HepG2 Cells*

After cells were cultured in glucose-free medium for 12, 24, 36, or 48 h, intracellular NO levels were found to decrease in a time-dependent manner. Glucose depletion increased NO production to 123.7 ± 3.0% at 12 h, but this was followed by a decrease in the intracellular NO level to 82.9 ± 2.6%, 72.9 ± 6.6%, and 46.1 ± 5.0% at 24, 36, and 48 h, respectively, compared with their respective control groups (P < 0.05; Fig. 1A). To further examine whether treatment of cells with glucose depletion reduced NO level, cells underwent glucose depletion for 0.5 to 48 h. Western blot analysis of the glucose depletion–treated cells indicated that there was a time-dependent decrease in endothelial NOS (eNOS) and neuronal NOS (nNOS) at the protein level (Fig. 1B). However, the protein expression of inducible NOS (iNOS) was not detectable at any time point (data not shown). We next asked whether a NO donor can block the decrease in NO level. We used 0.5 mmol/L sodium nitroprusside (SNP) to increase the NO level and our results showed that SNP increased the NO level under the glucose-free condition at 24 and 48 h compared with the glucose depletion condition, but there was only a slight decrease in NO level under high glucose at 24 and 48 h (Fig. 2A). Because NO is generated by NOS, we measured protein expression levels of the NOS by Western blot. Figure 2B shows that glucose depletion decreased the protein expression of nNOS and eNOS, and SNP blocked the decrease in eNOS and nNOS at the protein level.
The Effect of Glucose Depletion and NO on Cell Viability in Human Hepatoma HepG2 Cells

To further investigate the effect of drug treatment on the viability of cells, we measured cell viability using the lactate dehydrogenase (LDH) assay. Glucose depletion significantly decreased cell viability at 48 h. After treatment of cells from the glucose depletion group with two different NO donors, SNP and 8-bromo-cGMP, cell viability significantly increased compared with the untreated glucose depletion group at 48 h (P < 0.05; Fig. 3A). SNP or S-nitroso-N-acetyl-penicillamine treatment alone had no significant effect on cell viability at 48 h. Moreover, on preincubation of the cells with 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO), a stable radical scavenger for NO, in the presence or absence of the SNP for 48 h, PTIO significantly blocked the SNP cytoprotective effect (P < 0.05; Fig. 3A). In addition, we used another NO donor, S-nitrosothioglutathione, and found that it also had cytoprotective effect, which PTIO also significantly blocked (data not shown). NO often induces the activation of soluble guanylyl cyclase and intracellular signaling via cGMP (24). To determine if NO-protected glucose depletion–induced cytotoxicity was mediated by guanylyl cyclase or cGMP, we treated cells with 100 μmol/L 8-bromoguanosine 3',5'-cyclomonophosphate (8-bromo-cGMP), a cGMP analogue. 8-Bromo-cGMP had similar cytoprotective effect to the NO donors (Fig. 3B). Additionally, when cells were pretreated for 1 h with 100 μmol/L 1H-[1,2,4]oxadiazolo[4,3-a]-quinazolin-1-one, a selective inhibitor of soluble guanylyl cyclase, the SNP cytoprotective effect was blocked (Fig. 3B). Because LDH release can occur without complete loss of cell membrane integrity, we further used Annexin V/propidium iodide assay to confirm that cells were killed via apoptosis. Figure 3C shows that glucose depletion induced apoptotic cells, and NO cytoprotective effect is mediated through a cGMP-dependent pathway. Together, these results suggest that SNP prevention of glucose depletion–induced cell damage was mediated by a cGMP-dependent pathway.

SNP Blocked Glucose Depletion–Induced Mitochondrial Mass Change and Morphology Alteration in Human Hepatoma HepG2 Cells

Glucose is an important energy source for cancer cells and mitochondria are the major organelle for energy production in such cells; therefore, we next studied the effect of glucose depletion on changes in mitochondrial mass. To determine mitochondrial mass after glucose depletion and/or SNP treatment, we used the fluorescent dye 10-nonyl-acridine orange (NAO) to analyze the mitochondrial mass. The relative NAO intensity of the glucose-depleted cells was significantly lower than that of the control at 24 and 48 h (P < 0.05; Fig. 4A). In contrast, SNP and glucose depletion cotreatment of HepG2 cells increased NAO intensity (51.7 ± 7.7%) comparable with that of the glucose-free group at 48 h (P < 0.05; Fig. 4A). However, SNP treatment alone significantly decreased NAO intensity compared with the control. To further confirm the changes in mitochondrial mass after glucose depletion and/or SNP treatment, cells were stained with the fluorescent dye Mitotracker green and images were recorded by confocal microscopy. It was found that the mitochondria of the control cells showed a typical reticulum shape (Fig. 4B). However, the glucose depletion treatment of HepG2 cells decreased mitochondrial mass and the reticulum seemed to be disrupted and fragmented and there was a distribution of small individual organelles (Fig. 4B). The SNP treatment–only group had a similar morphology to that of control. Cotreatment of SNP with glucose depletion increased mitochondrial mass, and mitochondria showed a granular morphology (Fig. 4B). These findings suggest that SNP blocked glucose depletion–induced mitochondrial damage.

FIGURE 2. Effect of glucose depletion and SNP on the endogenous level of NO and NOS in human hepatoma HepG2 cells. A. Cells were glucose depleted (−G) or treated with 0.5 mmol/L SNP alone or SNP combined with glucose depletion for 24 or 48 h. NO level was detected by 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate fluorescence dye and analyzed by flow cytometry as described in Materials and Methods. The NO level in control cells was assigned the value of 100%. *P < 0.05, significant difference versus their respective control. #, P < 0.05, significant difference versus their respective glucose depletion group. B. The protein levels of eNOS and nNOS in HepG2 cells after 48 h treatment. β-Actin was used as a loading control.
The Effect of Glucose Depletion and SNP on the Endogenous Level of ATP in Human Hepatoma HepG2 Cells

Because mitochondrial mass in HepG2 cells showed variation after glucose depletion and/or SNP treatments, we further measured changes in ATP levels using an ATP bioluminescence assay kit. After treatment of HepG2 cells for 24 to 48 h with glucose depletion, the ATP level of the cells had decreased significantly at 24 and 48 h (P < 0.05; Fig. 5). SNP treatment alone had no significant effect at 24 and 48 h compared with the control. Simultaneous treatment of HepG2 cells with glucose depletion and SNP resulted in an increase in the ATP level at 24 and 48 h (Fig. 5).

The Effects of Glucose Depletion and SNP on the Copy Number of mtDNA and Peroxisome Proliferator-Activated Receptor γ Coactivator-1α mRNA Expression in Human Hepatoma HepG2 Cells

Because our data found that NO prevented glucose depletion–induced mitochondrial alteration, it is most likely that the glucose depletion reduced mtDNA copy number and the expression of peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α), a cofactor for mitochondrial biogenesis. Using real-time PCR analysis, we found that glucose depletion was able to decrease mtDNA copy number and PGC-1α mRNA expression in a time-dependent manner (Fig. 6A). Furthermore, SNP inhibited the decrease, under glucose depletion, of the mtDNA copy number and of the PGC-1α mRNA level at 24 h (P < 0.05; Fig. 6B). However, SNP treatment alone only showed a slight decrease in mtDNA copy number compared with the control (Fig. 6B). Our results further confirmed that SNP blocked the glucose depletion–induced changes in mtDNA and PGC-1α mRNA.

An Analysis by Western Blotting of the Effect of Glucose Depletion and SNP on Protein Expression

We next investigated the effects of glucose depletion and/or SNP on the amount of mitochondrial complex proteins by Western blot at 48 h. Glucose depletion decreased complex I, complex III, heat shock protein 60 (Hsp60), and cytochrome

FIGURE 3. Effect of NO and cGMP on glucose depletion–induced cytotoxicity in human hepatoma HepG2 cells. A. Cells were treated with 0.5 mmol/L SNP or 0.25 mmol/L S-nitroso-N-acetyl-penicillamine (SNAP) in the presence or absence of glucose. Cells were pretreated for 1 h with 100 μmol/L PTIO, followed by exposure to SNP with or without glucose for 48 h. B. The cell survival rate was determined by LDH assay as described in Materials and Methods. Cells were treated with 0.5 mmol/L SNP or 100 μmol/L 8-bromo-cGMP (8-Br-cGMP) with or without glucose for 48 h. Cells were pretreated with 100 μmol/L 1H-[1,2,4]oxadiazolo[4,3-a]quinolin-1-one for 1 h, followed by exposure to SNP in the presence or absence of glucose for 48 h. The responses of cells to PTIO, 1H-[1,2,4]oxadiazolo[4,3-a]quinolin-1-one, 8-bromo-cGMP, S-nitroso-N-acetyl-penicillamine, or SNP alone were also monitored. C. Effect of NO and cGMP on glucose depletion–induced apoptosis in human hepatoma HepG2 cells. The drug treatment is the same with B. *, P < 0.05, a significant difference versus their respective control. #, P < 0.05, a significant difference versus their respective glucose depletion group. Columns, mean from three independent experiments with triplicate samples; bars, SD.
c protein levels, but had no significant effect on complex II, complex IV subunit I (cyclooxygenase I), complex IV subunit II (cyclooxygenase II), and complex V (ATPase) protein levels (Fig. 7). SNP treatment alone had no significant effect compared with the glucose treatment–alone group (Fig. 7).

The glucose depletion–induced decrease in complex I, complex III, cytochrome c, and Hsp60 was blocked when SNP was added during glucose depletion.

Rotenone and Antimycin A Blocked SNP-Induced Effects during Glucose Depletion Condition

Finally, we attempted to examine if mitochondria are involved in the SNP block of glucose depletion–induced cytotoxicity. Because our Western blot results showed that glucose depletion specifically decreased the protein level of complex I and complex III in mitochondria, we used a mitochondrial complex I inhibitor, rotenone, to block complex I activity and the complex III inhibitor, antimycin A, to block complex III activity for 48 h. Rotenone in the presence of glucose and/or SNP had no significant effect on cell survival (Fig. 8A). However, the SNP protection effect on HepG2 cells was blocked when rotenone was added during glucose depletion (Fig. 8A). Figure 8B shows that the effect of antimycin A was similar to that of rotenone. These results together suggested that mitochondrial activity is essential for the SNP protection effect during glucose depletion–induced cytotoxicity.

Discussion

Our data showed that NO protects against glucose depletion–induced cytotoxicity in hepatoma cells by increasing mitochondrial biogenesis. We observed that glucose deprivation lead to a decrease in cellular NO level and this was accompanied by changes in the level of eNOS and nNOS protein expression. The NO donor SNP not only blocked the glucose depletion–induced decrease in NO but also protected

FIGURE 4. Effect of glucose depletion and SNP on the level of mitochondrial mass in human hepatoma HepG2 cells. A. Cells were glucose depleted or treated with 0.5 mmol/L SNP alone or in combination with SNP under glucose depletion for 24 or 48 h. Mitochondrial mass was detected using the NAO fluorescent dye and analyzed by flow cytometry as described in Materials and Methods. NAO intensity in control cells was assigned the value of 100%. Representative histograms of cells stained with NAO at 24 and 48 h. B. After a 24-h treatment, cells from control, glucose-free, and 0.5 mmol/L SNP treatment with or without glucose groups were observed under a confocal microscope after the cells were stained with Mitotracker green dye. *, P < 0.05, significant difference versus their respective control. #, P < 0.05, significant difference versus their respective glucose depletion group. Columns, mean from three independent experiments with duplicate samples; bars, SD.

FIGURE 5. Comparison of glucose depletion and SNP on the intracellular ATP content and mitochondrial membrane potential in human hepatoma HepG2 cells. ATP level was determined by the ATP bioluminescence assay as described in Materials and Methods. Cells were glucose depleted or treated with 0.5 mmol/L SNP alone or SNP combined with glucose depletion for 24 or 48 h. The ATP level in the control cells was assigned the value of 100%. *, P < 0.05, significant difference versus their respective control. #, P < 0.05, significant difference versus their respective glucose depletion group. Columns, mean from three independent experiments with duplicate samples; bars, SD.
hepatoma cells from glucose depletion–induced cytotoxicity. In addition, mitochondrial inhibitors blocked the SNP cytoprotection function. These results clearly showed that the SNP protection against glucose depletion–induced cytotoxicity is mediated through mitochondria.

It is well known that NOS are key enzymes in the production of NO. Previous studies have shown that glucose depletion decreases NO production and increases cell death in immunostimulated rat primary astrocytes. However, no change was found in the mRNA and protein levels of iNOS (25). It has also been shown that eNOS and nNOS are constitutively expressed and calcium dependent, but iNOS is a calcium-independent and cytokine inducible isofrom (26). Moreover, previous studies have shown that glucose depletion decreases cytoplasmic calcium concentration (27) and NO donors, nitroglycerin, and SNP increase calcium uptake in rat heart and liver mitochondria (28). In the present study, glucose depletion induced a decrease in NO in hepatoma cells (Fig. 1), but no iNOS could be detected. These results together suggest that glucose depletion and the NO donor together may have affected the intracellular calcium concentration of the hepatoma cells and this resulted in a modulation in the expression of eNOS and nNOS protein, but not of iNOS.

Energy production is an essential factor for cell survival, and the citric acid cycle is an important energy production reaction in mitochondria. Under the glucose depletion condition, cancer cells can obtain energy supply using alternative energy substrates such as fatty acids and amino acids for glutaminolysis and β-oxidation (29, 30). In addition, changes in mitochondrial structure and oxidative phosphorylation activity have been observed in HeLa cells under glucose depletion. Moreover, NO activates mitochondrial biogenesis in various cancer cell lines (23). These results agree well with our observation that NO maintained mitochondrial biogenesis (Fig. 4) and function (Fig. 5) under a glucose-free condition. Because glucose was depleted in the medium, the NO-blocked glucose depletion–decreased ATP most likely was the result of an alternative energy supply from amino acids and fatty acids via the citric acid cycle. Taken together, NO seems to augment the cellular ATP level and served as a protector during glucose depletion–induced damage in hepatoma cells.

NO has been reported to compete with oxygen when binding to cytochrome c oxidase, and this leads to an inhibition of mitochondrial respiration and a decrease in mtDNA copy number (31-33). On the other hand, Nisoli et al. (22, 23) showed that NO-induced mitochondrial biogenesis and expression of genes encoding mitochondrial proteins via cGMP-dependent pathway seems to be linked to proliferation and differentiation in normal and tumor cells. The significance of this change in energy metabolism remains to be investigated. Our results indicated that cytoprotective effect of SNP is cGMP dependent (Fig. 3B and C), so the cytoprotective effect of the cGMP pathway stimulation by NO seems to outweigh the
direct inhibitory effect of NO on mitochondrial cytochrome c oxidase. Consequently, the NO-stimulated cGMP pathway seems to be important for mitochondrial preservation and NO-mediated cytoprotection under glucose depletion conditions.

Thus, NO-induced mitochondrial biogenesis may vary across different diverse cell types and between normal and tumor cells. NO may play a dual role in regulating mitochondria. Previous studies on NO regulation of mitochondria were carried out under high glucose condition; however, cells may respond to energy limitation and therefore we compared the NO-induced mitochondrial changes in the presence and absence of glucose. Our results showed that there was a NO-inhibitory effect on the glucose depletion–induced decrease in mtDNA copy number and loss of PGC-1α mRNA, but that there was no significant effect on mitochondrial biogenesis and function under high glucose (Fig. 6). In several cell lines and tissues, recent studies have shown that NO produced by eNOS-generated mitochondrial biogenesis acts through the activation of PGC-1α and produced an increase in mtDNA and mitochondrial proteins (22, 34). Moreover, a recent study indicated that NO treatment of endothelial cells upregulated PGC-1α, which was also mediated through cGMP pathway (35). We found that SNP blocked the glucose depletion decrease in mtDNA copies and PGC-1α mRNA. Thus, it is possible that SNP may activate PGC-1α to generate mitochondrial biogenesis. This observation agrees well with findings that mitochondria are the key target during NO stress.

Interestingly, we have found that SNP was able to maintain mitochondrial biogenesis and function under glucose depletion condition. We use rotenone and antimycin A to inhibit the NO protection effect (Fig. 8). We also found that HepG2 cells pretreated for 10 days with a low concentration etidium bromide, which produces a decrease in mtDNA, or for 2 days with chloramphenicol, which produces a decrease in mtDNA-encoded proteins, blocked the SNP cytoprotection effect (data not shown). These results clearly showed that the NO cytoprotective effect is mediated by mitochondrial function. Therefore, SNP-maintained mitochondrial biogenesis and function under glucose depletion is an essential factor for protection against glucose depletion–induced cytotoxicity.

A recent study showed that mitochondrial structure changes when HeLa cells are cultured in a galactose medium that restricts glucose uptake (29). Glycolysis is blocked in the galactose medium, and HeLa cells metabolically adapt to the energy source by increasing mitochondrial proteins and oxidative capacity. In this study, we have shown that Hsp60, a specific mitochondrial heat shock protein, decreased under the glucose depletion condition (Fig. 7), and the change in Hsp60 agrees well with reduced mitochondrial mass (Fig. 4). Furthermore, confocal images showed the presence of a disrupted mitochondrial network under glucose depletion condition in hepatoma cells. Based on Western blot results for Hsp60, mitochondrial complex I and mitochondrial complex III, and NAO analysis together with the confocal images, we clearly have shown that glucose depletion reduced mitochondrial biogenesis and SNP inhibited this effect.

The tumor microenvironment is recognized to be a major factor associated with tumor progression and this affects anticancer therapy (5). A recent study has shown that the tumor environmental pH affects cytokine-mediated death in a L929 fibrosarcoma cell line and C6 glioma cell lines through modulation of iNOS-dependent NO synthesis in both tumor cells and macrophages (36). They showed that acidosis inhibited lipopolysaccharide and cytokines induced NO and blocked NO-induced cytotoxicity. However, it is interesting that pH or acidosis has no effect on the level of exogenous NO generated by SNP treatment. NO-induced cytotoxicity in cancer cells and NO-induced survival in hepatoma cells are opposite. One possibility is that NO plays a different role in different cell types. Alternatively, it is possible that the endogenous and exogenous NO may have different functions and tumor cells may show differential responses to NO variation. Modulation of NO within the tumor microenvironment is one possible approach to anticancer therapy. Additionally, energy boost therapy is a new area of interest in anticancer therapy (37), and it has been suggested that dietary energy restriction is a potent inhibitor of carcinogenesis and tumor cell growth (38). Our results indicated that NO acts against glucose depletion–induced cytotoxicity, so it may be important during tumor treatment to modulate NO level, especially when under glucose depletion condition and thus a combination of NO scavengers or NOS inhibitors together with energy restriction may be an effective treatment of cancer.

In conclusion, our results showed that NO blocked the glucose depletion–induced effect via modulation of

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**FIGURE 7.** Effect of glucose depletion and SNP on protein expression as analyzed by Western blot. The protein levels of mitochondrial complexes, Hsp60, and cytochrome c in HepG2 cells were determined at 48 h after treatment. β-Actin was used as a loading control.
mitochondrial biogenesis and function. Although the detailed mechanism of NO protection of cells under energy depletion needs further investigation, modulation of NO in a poor nutrient environment may provide clues that can lead to new combinatorial cancer therapy.

Materials and Methods

Cell Culture

HepG2, a well-differentiated human hepatoma cell line originally isolated from hepatoblastoma (39), was cultured in DMEM with 25 mM D-glucose supplemented with 10% fetal bovine serum, 10 μg/mL gentamicin, 2 mM/L L-glutamine, and 10 mM/L nonessential amino acid at 37°C in a humidified atmosphere containing 5% CO2. Glucose depletion was achieved by washing the cells with HBSS once and the medium was changed to DMEM without glucose supplemented with 10% fetal bovine serum, 10 μg/mL gentamicin, 2 mM/L L-glutamine, 10 mM/L nonessential amino acid, and 1 mM/L sodium pyruvate (all culture reagents were purchased from Life Technologies).

Cell Viability Assay (LDH Assay)

The LDH levels were determined using a CytoTox 96R nonradioactive cytotoxicity assay (Promega). We evaluated cell death and cell growth at 48 h after application of 0.5 mM SNP (Sigma-Aldrich), 0.25 mM L-S-nitroso-N-acetyl-penicillamine (Sigma-Aldrich), 100 mM/L rotenone (Sigma-Aldrich), 100 mM/L antimycin A (Sigma-Aldrich), or 100 μM/L 8-bromo-cGMP (Sigma-Aldrich) or 0.25 mM/L L-S-nitrosoglutathione (Sigma-Aldrich) at 48 h in the presence or absence of glucose. Cells were pretreated with 100 μM/L 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (Calbiochem) or 100 μM/L PTIO (Sigma-Aldrich) for 1 h before being incubated with SNP and/or glucose depletion for 48 h. The assay measures the LDH released into the culture medium over a 20-min coupled enzymatic reaction at room temperature. The product was detected at 490 nm and used as a measure of LDH release.

Annexin V Binding Assay

To determine the extent of apoptosis, the Annexin V-FITC kit (Serotec) was used. Cells (2 × 10^5) were suspended in 195 μL binding buffer with 5 μL of Annexin V–FITC solution and incubated for 10 min in the dark at room temperature. Cells were washed and resuspended in 190 μL binding buffer with 10 μL of propidium iodide and analyzed by flow cytometry. The presence of viable (Annexin V negative and propidium iodide negative), early apoptotic (Annexin V positive and propidium iodide negative), late apoptotic (Annexin V positive and propidium iodide positive), and necrotic (Annexin V negative and propidium iodide positive) cells were analyzed by flow cytometry. The percentage of apoptosis in this experiment was quantified as percentage of Annexin V–positive with propidium iodide–positive and Annexin V–positive with propidium iodide–negative cells.

Determination of Mitochondrial Mass

The fluorescent dye NAO (Molecular Probes) was used to measure mitochondrial mass. Cells were trypsinized and resuspended in 0.5 mL PBS containing 10 μM/L NAO. After incubation for 10 min at room temperature in the dark, cells were transferred immediately to tubes on ice for flow cytometric analysis. The FACScan flow cytometer has an argon laser with excitation at 488 nm and emission was recorded at 530 nm. The data were analyzed by Cell Quest (Becton Dickinson) and were expressed as the mean fluorescence of NAO.

Confocal Image

Cells grown on coverslips in Petri dishes (Corning) in phenol red–free DMEM were stained with Mitotracker green (Molecular Probes) at 37°C for 30 min. A confocal microscope (Zeiss Inverted Axivert 200M) with a dip in lens Plan Fluor objective (×40 water) was used. The excitation was set at 488 nm (argon-ion tunable IR laser), and emission was recorded at 520 nm. Images were acquired using LSM 5 PASCAL software.

Diaminofluorescein Staining Analysis of NO

NO was measured using the probe 4-amino-5-methylamino-2′,7′-difluoroisoflourescein diacetate (Molecular Probes). Cells

![FIGURE 8. Mitochondrial inhibitors blocked the SNP-induced effect. A. Viability was measured by LDH assay, after 48 h treatment of 100 mM/L rotenone with or without 0.5 mM/L SNP in the presence or absence of glucose. B. Viability was measured by LDH assay after 48 h treatment of 100 mM/L antimycin A with or without 0.5 mM/L SNP in the presence or absence of glucose. *, P < 0.05, significant difference versus their respective glucose-only group. Columns, mean from three independent experiments with duplicate samples; bars, SD.](image-url)
were incubated with 5 mmol/L 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate in culture medium for 30 min at 37°C in the dark. At the end of incubation, the cell suspensions were kept in an ice bath (4°C). Propidium iodide (10 μg/mL, Sigma-Aldrich) was added to samples for gating the viable cells containing 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate and the cells were kept on ice for 10 min. The fluorescence intensity was quantified using a flow cytometer (Becton Dickinson) with excitation wavelength at 488 nm and emission wavelength at 530 nm. The red fluorescence (propidium iodide) was collected through a 585-nm filter. Data were analyzed by Cell Quest (Becton Dickinson) and were expressed as mean fluorescence of 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (40).

Measurement of ATP Concentration
The ATP concentrations in the cells were determined using an ATP Bioluminescence Assay kit (Roche Applied Science) based on the light-emitting luciferase-catalyzed oxidation of luciferin.

Determination of mtDNA Copy Number
Total DNA was extracted from HepG2 cells using the QIAamp DNA Mini Kit (Qiagen Technologies) according to the instructions of the manufacturer. The mtDNA copy number was determined using a real-time quantitative PCR method. Real-time quantitative PCR was done using the ABI PRISM 7700 (PE Applied Biosystems) and results were analyzed with the accompanying software. The sense primer for mtDNA was 5'-CACCCA-AGAACAGGGTTTGT-3' and the antisense primer was 5'-TGGCATGGAATTTGCTAA-3'. The sense primer for β-actin was 5'-ACCACACTGTGCCCATCTAC-3' and the antisense primer was 5'-TCGGTGAGGATCTTCATGAGGA-3'.

The DNA-intercalating SyBr green reagent was used for detection of the PCR product. The PCR cycle used was as follows: 50°C (2 min), 95°C (10 min), followed by 40 cycles of 95°C (15 s), 60°C (1 min). The level of gene expression was obtained using the ΔΔCt method in which all samples are first normalized to the level of β-actin in each sample. Relative normalized units were then compared between the control and treatment groups.

Quantitative Real-time Reverse Transcription-PCR Analysis
Total RNA was extracted from tissues using the TRIzol reagent (Life Technologies) according to the instructions of the manufacturer. The first-strand cDNA was synthesized with a random primer pd(N)6 in a 33°C mixture using reverse transcription beads (Amersham Pharmacia Biotech). Real-time quantitative PCR was done using the ABI PRISM 7700 PE Applied Biosystems) and results were analyzed with the accompanying software. The sense primer for PGC-1 was 5'-CCAAATGGACCCCAAGGTTCC-3' and the antisense primer was 5'-TATGAGGAGGATGGGTTGTC-3'. The sense primer for β-actin was 5'-ACCACACTGTGCCCATCTACTAC-3' and the antisense primer was 5'-TCGGTGAGGATCTTCATGAGGTA-3'.

The RNA-intercalating SyBr green reagent was used for detection of the reverse transcription-PCR product. The PCR cycle used was as follows: 50°C (2 min), 95°C (10 min), followed by 40 cycles of 95°C (15 s), 60°C (1 min). The level of gene expression was obtained using the ΔΔCt method in which all samples are first normalized to the level of β-actin in each sample. Relative normalized units were then compared between the control and treatment groups.

Western Blot Analysis
Cells were plated at a density of 1 × 10^6 per 100-mm dish and were harvested at 48 h after treatment. Whole-cell lysate was prepared by resuspending the cells in M-PER protein extraction reagent (Pierce) supplemented with protease inhibitors cocktail (Pierce) and incubating the cells on ice for 30 min. Then, the cell lysates were centrifuged at 12,000 × g for 15 min and the supernatant was collected. Protein concentration was measured using a BCA protein assay kit (Pierce). An aliquot of protein lysate (30 μg) from each sample was mixed with 2 × Laemmli sample buffer (Bio-Rad), and protein lysate was separated on a 10% SDS-polyacrylamide gel for 1 h. After separation, the proteins were transferred to a nitrocellulose membrane and the membrane was blocked with 5% milk in 1 × TBS buffer [10 mmol/L Tris, 150 mmol/L NaCl, 0.5% Tween 20 (pH 7.4)] for 1 h at room temperature. The proteins were probed with antibodies against eNOS, nNOS, iNOS (BD), complex I (α subcomplex, 9), complex II (flavoprotein), complex III (core I subunit), cytochrome-c oxidase I (complex IV subunit I), cytochrome-c oxidase V (complex IV subunit IV), complex V (β subunit; Molecular Probes), Hsp60, cytochrome-c (Santa Cruz Biotechnology, Inc.), and actin (Sigma) at 4°C overnight and this was followed by incubation with horseradish peroxidase–conjugated secondary antibodies (Sigma). Protein visualization was carried out using enhanced chemiluminescence kit (Pierce) according to the manufacturer’s protocol.

Statistical Analysis
Statistical analyses were done using SigmaPlot software. Comparisons between the mean of various treatment groups were analyzed using Student’s t test. Data are presented as mean ± SD except where indicated. The difference was considered significant when P < 0.05.

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References
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