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Original Contribution

NITRIC OXIDE AND BCNU CHEMORESISTANCE IN C6 GLIOMA CELLS: ROLE OF *S*-NITROSOGLUTATHIONE

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Abstract—Inducible nitric oxide synthase (iNOS or NOS2) is expressed in malignant glioma. Previously we noted that C6 glioma cells overexpressing NOS2 displayed chemoresistance against 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and other chloroethylnitrosourea derivatives with carbamoylating action. Herein we report experimental evidence supporting the contention that this NOS2 effect is mediated, at least in part, by *S*-nitrosoglutathione (GSNO), a potent antioxidant derived from interaction of NO and glutathione. Out of three NO donors tested, only GSNO was effective in protecting glioma cells against BCNU cytotoxicity. Furthermore, the protective effect of GSNO, similar to that of NOS2, was confined to carbamoylating, but not alkylating action. Experimental manipulations that were expected to increase or decrease cellular GSNO stores, as confirmed by immunocytochemical staining using a GSNO-specific antibody and HPLC analysis of GSNO contents in culture medium, led respectively to enhanced or reduced chemoresistance against carbamoylating cytotoxicity. Finally, neocuproine, a selective cuprous ion chelator known to neutralize GSNO actions, abolished NOS2-mediated chemoresistance against carbamoylating agents. Our results reveal a novel action of NOS2/GSNO that may potentially contribute to the development of chemoresistance against BCNU, which remains a mainstay in chemotherapy for glioblastoma multiforme.

Keywords—Alkylation, Carbamoylation, Inducible nitric oxide synthase, Malignant glioblastoma multiforme, *N*-Acetyl-L-cysteine, Nitric oxide, *S*-Nitrosoglutathione, *S*-Nitrosothiol, Free radicals

INTRODUCTION

Glioblastoma multiforme (GBM) is the most common type of primary brain tumor, accounting for more than 40% of neoplasms in the central nervous system (CNS) [1]. Compared with other cancers, the life expectancy of patients with GBM is relatively short. The combination of surgery, radiotherapy, and chemotherapy results in a survival of approximately 14 months [2]. 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU) is the mainstay in chemotherapy of GBM, due in part to its lipophilic character, which allows better passage across the blood–brain barrier [3]. Unfortunately, BCNU does not appear to substantially prolong median survival, even though the proportion of patients living more than 18 months increased from 5 to 15% with adjunct BCNU chemotherapy [4–6]. The mechanism of GBM resistance to BCNU chemotherapy remains to be fully delineated. Variations in multidrug resistance genes [7], DNA repair activity such as *O*6-methylguanine-DNA methyltransferase [8], and glutathione *S*-transferase and intracellular glutathione content [9] have all been speculated to cause BCNU chemoresistance.

Nitric oxide (NO) is a free radical gas mediating a number of physiological functions including modulation of vascular tone, neural transmission, and cell viability. NO is synthesized from arginine and oxygen and catalyzed by nitric oxide synthases (NOS). Inducible NOS (iNOS or NOS2) has been shown to cause excessive formation of NO in pathological conditions such as inflammation [10]. Expression of NOS2 has been demonstrated in human glioma [11] and in a variety of brain

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tumors or peritumor areas, with its mRNA levels higher in malignant glioma than normal brain tissues [12] or meningioma [13]. NO may contribute to antioxidant action via its interaction with glutathione to form *S*-nitrosoglutathione (GSNO), an antioxidant that is two orders of magnitude more potent than the reduced form of glutathione (GSH) [14]. At micromolar concentrations, GSNO is capable of neutralizing oxidative stress exerted by peroxynitrite [15], which is a highly reactive species derived from interaction of NO with superoxide anions [16].

Chloroethylnitrosoureas such as BCNU and 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) kill tumor cells via multiple cytotoxic actions including carbamoylation and alkylation [17]. In an earlier study, we noted that overexpression of NOS2 in rat C6 glioma cells enhances chemoresistance against the carbamoylating, but not alkylating, action of BCNU and related chloroethylnitrosourea derivatives [18]. In the present study, we report that GSNO may mediate NOS2-induced chemoresistance against carbamoylating agents in C6 glioma cells in vitro.

MATERIALS AND METHODS

Materials

All the chemicals were purchased from Sigma (St. Louis, MO, USA) unless otherwise specified. BCNU was from Bristol–Myers Squibb Inc. (Princeton, NJ, USA). Temozolomide was a gift from Dr. W. Robert Bishop at Schering–Plough Corporation (Kenilworth, NJ, USA). The three 1,2-bis(sulfonyl)hydrazine derivatives including 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)-hydrazine (compound 1), 1,2-bis(methylsulfonyl)-1-[[(2-chloroethyl)amino]-carbonyl]-hydrazine (compound 3), and 1,2-bis(methylsulfonyl)-1-[[(methyl)amino]carbonyl]-hydrazine (compound 5) were generously provided by Dr. Alan C. Sartorelli, Department of Pharmacology, Yale University School of Medicine. The synthesis and characterization of compounds 1, 3, and 5 have been described in detail elsewhere [19,20].

Cell culture and transient gene transfer

Rat C6 glioma cells (American Type Culture Collection, Rockland, MD, USA) were grown in HAM's F-12 medium (BioWhittaker, Walkersville, MD, USA) supplemented with 15% horse serum (BioWhittaker), 2.5% fetal bovine serum (BioWhittaker), 0.15% sodium bicarbonate, 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Grand Island, NY, USA). Transient transfection of a NOS2-expressing construct, NS05 (Oxford Biomedical Research, Oxford, MI, USA), and the negative control vector pcDNA3.1 (Invitrogen, Carlsbad, CA, USA) in 24-well culture vessels using SuperFect (Qiagen Inc., Valencia, CA, USA) was performed according to the manufacturers' instructions with a ratio of DNA to SuperFect reagent of 1:2 for 3 h. DNA/SuperFect mixtures were then removed and the cells were washed twice with phosphate buffered saline (PBS) before fresh medium was applied. Cells were cultured for an additional 24 h to allow NOS2 expression. Successful transfection of NS05 causing NOS2 overexpression in C6 glioma cells has been previously confirmed by Western blotting and by an increase in the nitrite content based on the Griess reaction [18]. The empty vector pcDNA3.1 was separately transfected to serve as negative controls. In the present study, NOS2 overexpression was confirmed by an increase in the medium nitrite content based on the Griess reaction.

Drug treatments

The transfected NOS2 gene was allowed to express for 24 h before the transfectants were challenged with chemotherapeutic drugs for an additional 12 to 48 h, depending on the agent used. In selected experiments where cytotoxicity was examined following alteration of cellular glutathione levels, cells were pretreated with *N*-acetyl-L-cysteine (NAC), a glutathione precursor for 2 h or buthionine sulfoximine (BSO, 125 μM), an irreversible inhibitor of γ-glutamylcysteine synthetase that depletes cellular glutathione stores, for 18 h before exposure to chemotherapeutic agents. In selected experiments in which the long-term effects of low-dose chemotherapeutic agents were tested to simulate clinical situations, cells at lower densities (less than 10%) were treated with cyclohexyl isocyanate or temozolomide with or without GSNO for 5 days. Culture medium containing the drugs was replenished every 24 h until the end of the experiments when cell viability was assessed.

Nitrite assay, GSNO measurement, and immunocytochemistry

The extent of NO formation was assessed by using the Griess reaction to measure the amount of nitrite released into the medium as reported previously [18,21]. The GSNO content of medium was quantitatively determined by HPLC as described [22]. Briefly, culture medium was filtrated through a filter disk with a pore size of 0.22 μm and then 10 μl of filtrated medium was diluted with 90 μl of phosphate butter (50 mM, pH 7.4). The diluted samples were then injected into a reverse phase C-18 column (250 × 4.6 mm) at a flow rate of 1 ml/min with a total running time of 13 min. The composition of the mobile phase solvent was 10% acetonitrile and 90% sodium dihydrogen phosphate (10 mM) containing 1-octanesalfonic acid (10 mM) in HPLC-grade water. The signal was detected at

208 nm. GSNO purchased from Sigma was spiked as the standard.

For immunofluorescence staining of cellular GSNO, cells after various experimental manipulations were fixed with 4% formaldehyde in 1× PBS for 20 min. A mouse monoclonal antibody against GSNO (A.G. Scientific, Inc., San Diego, CA, USA) was then applied at 1:2000 for 16 h at 4°C to stain the intracellular GSNO after fixation. The fluorescein-conjugated goat anti-mouse IgG secondary antibody (Molecular Probes, Eugene, OR, USA) was applied at 10 μg/ml for 1 h to recognize the GSNO primary antibody. The immunostained cultures were monitored under a Nikon vFM fluorescence microscope. Images were acquired with the B-2A filter. Four fields were randomly selected in each experimental condition for counting GSNO-positive cells.

Cell viability assays

For quantitative measures of the extent of cell survival or death, MTT and trypan blue exclusion assays were performed as previously described [18,23].

Statistical analysis

Results are expressed as means ± SD. Statistical analysis was performed using Student's unpaired *t* test between two experimental groups. Multiple groups were analyzed by one-way analysis of variance (ANOVA) followed by a post hoc Student–Newman–Keuls test. A *p* value less than .05 was considered significant.

RESULTS

Effects of NO donors on BCNU chemoresistance

We have previously shown that overexpression of NOS2 in C6 glioma cells leading to increases in the medium nitrite content confers chemoresistance against BCNU and other chloroethylnitrosoureas with carbamoylating action [18]. Based on this study, we assumed that an increase in cellular NO content to a similar magnitude would result in approximately the same degree of chemoresistance against BCNU. Thus, various concentrations of three NO donors, namely, GSNO, SNAP, and SIN-1, were tested in attempts to achieve cellular NO levels that would be comparable to that achieved by NOS2 overexpression to induce BCNU chemoresistance. We noted an increase in medium nitrite content after exposure of C6 cells to these three NO donors (Fig. 1A), reflecting probable increases in the cellular NO levels. Despite the finding that medium nitrite content was increased to a similar extent by these NO donors, their relative potencies in conferring chemoresistance against BCNU were variable (Fig. 1B). Only GSNO at 100 and 150 μM conferred chemoresistance against BCNU cytotoxicity

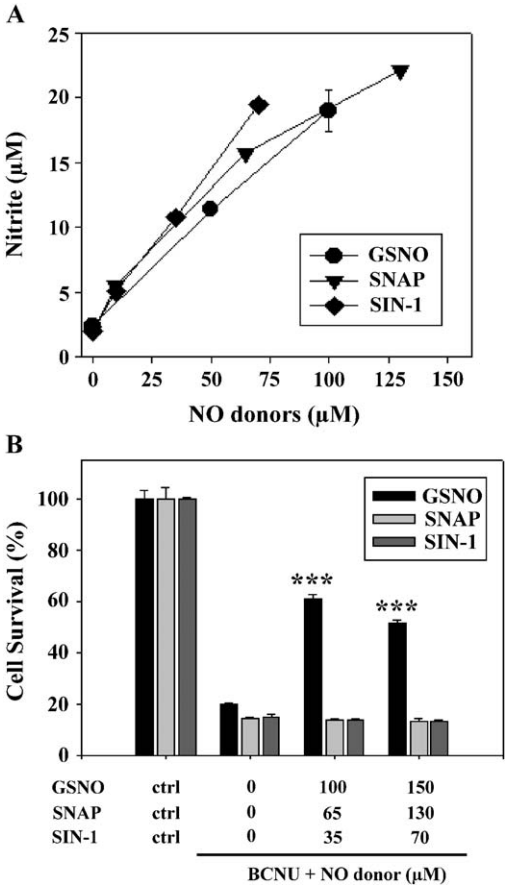

Fig. 1. Effect of NO donors on BCNU chemoresistance. (A) Nitrite levels, as determined by the Griess reaction, in the medium from cells treated with GSNO, SNAP, or SIN-1 at the indicated concentrations for 12 h. (B) Percentage survival of cells exposed to 0.47 mM (100 μg/ml) BCNU along with GSNO, SNAP, or SIN-1 at the indicated concentrations for 12 h. Cell survival was measured by the MTT assay. ***$p < .001$, significant difference as compared with the cells treated with BCNU but without GSNO. Data shown are mean ± SD and are representative of three separate experiments in triplicate samples with similar results.

(Fig. 1B). As NOS2 transfection occurred prior to BCNU exposure [18], we also tested whether pretreatment with the NO donors was required for their actions. When C6 glioma cells were pretreated with these three reagents followed by BCNU exposure, we found SNAP, another *S*-nitrosothiol that also release NO, afforded protection; SIN-1 was, however, completely ineffective (data not shown).

Effects of GSNO on carbamoylating and alkylating agents

BCNU exerts its tumoricidal effects by both carbamoylating and alkylating actions. The NOS2-induced chemoresistance, however, is confined only to carbamoylating cytotoxicity of BCNU [18]. We therefore tested whether the GSNO effect against BCNU toxicity was also restricted to the carbamoylating action. GSNO dose-

dependently attenuated cytotoxicity against compound 5, an agent carrying exclusively carbamoylating activity [19,20], based on both MTT and trypan blue exclusion assays (Fig. 2A). Similar effects of GSNO were observed with compound 3, another chloroethylnitrosourea analog with carbamoylating action [19,20], as well as cyclohexyl isocyanate and 2-chloroethyl isocyanate, the respective carbamoylating metabolites of CCNU and BCNU (data not shown). In contrast to its potent neutralizing effect against the carbamoylating agents, GSNO up to 400 μM did not confer chemoresistance against alkylating agents including compound 1 and temozolomide (Fig. 2B). Indeed, GSNO at concentrations greater than 400 μM caused dose-dependent cell death irrespective of the presence of chemotherapeutic reagents (data not shown).

The results presented in the previous [18] and current studies suggest that both NOS2 expression and exogenous application of GSNO protect C6 glioma cells specifically against carbamoylating, but not alkylating, agents. However, high concentrations (millimolar) of these chemotherapeutic agents were used in short-term experiments (12–48 h) in our studies, which do not mimic clinical situations. To address this concern, we treated C6 glioma cells at low densities (less than 10% confluence) with a lower concentration (500 μM) of the chemotherapeutic agents for an extended period (up to 5 days) in the presence and absence of GSNO. At a lower dose with extended exposure, GSNO was still effective in conferring chemoresistance in C6 glioma cells against carbamoylating agents including cyclohexyl isocyanate, but not temozolomide, which is an alkylating agent (Fig. 2C).

Immunofluorescence detection of intracellular GSNO and HPLC analysis of GSNO level in culture medium

GSNO is derived from interaction between NO and glutathione [24]. Heightened NO contents and/or enriched glutathione stores may increase cellular GSNO level to an extent that is detectable by immunofluorescence staining using a GSNO-specific antibody. Conversely, depletion of glutathione stores may lead to a lower cellular GSNO concentration that is below the detection limit of immunostaining. We therefore examined whether manipulations of NO and/or glutathione contents in C6 glioma cells may correspondingly alter the numbers of cells positively stained with the GSNO antibody based on immunofluorescence studies. Morphological examination under a phase-contrast microscope demonstrated that, under these experimental conditions, total numbers of the cells did not change significantly (data not shown). We found that induction of NOS2 by cytokine exposure (Fig. 3B) or pretreatment with *S*-nitrosothiols,

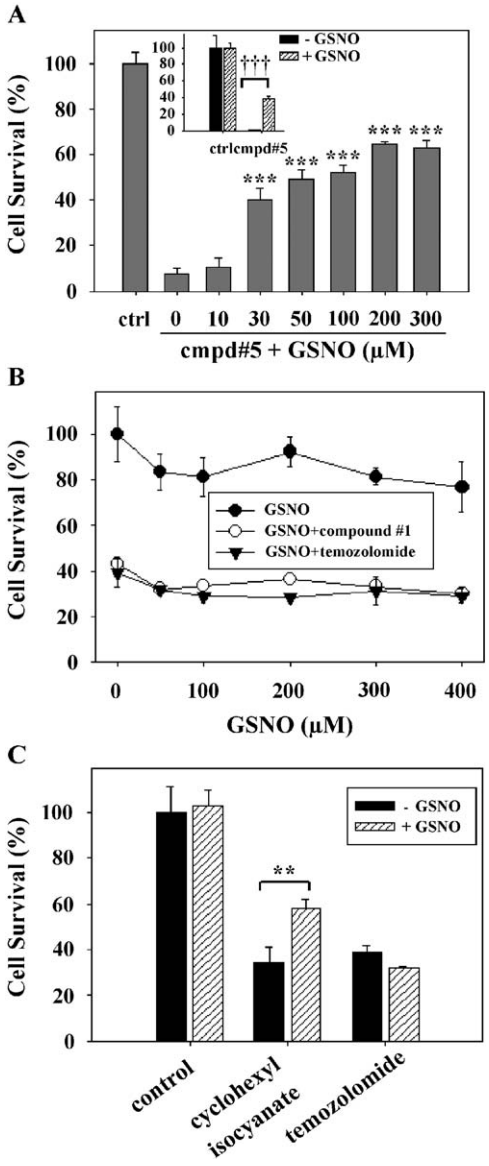

Fig. 2. Effects of GSNO on chemoresistance against carbamoylating and alkylating agents. (A) C6 glioma cells were exposed to 0.8 mM compound 5 without or with GSNO at the indicated concentrations for 24 h. ***$p < .001$, significant difference from samples exposed to compound 5 only. *ctrl*, cells without any treatment. Cell survivals were all based on the MTT assay. Inset: cell survival as determined by the trypan blue exclusion assay with (*hatched bars*) or without (*solid bars*) 100 μM GSNO. $^{\dagger\dagger\dagger}p < .001$. (B) Cells were exposed to 3 mM compound 1 (*open circles*) or 3 mM temozolomide (*solid triangles*) along with GSNO at the indicated concentrations for 48 h. Cells treated with GSNO only (*solid circles*) served as negative controls. Note that GSNO up to 400 μM did not alter chemoresistance against compound 1 or temozolomide. (C) Cells were exposed to 500 μM cyclohexyl isocyanate or 500 μM temozolomide in the absence (*solid bars*) or presence (*hatched bars*) of 100 μM GSNO for 5 days, with fresh medium containing the drugs replenished every 24 h. Control cells were without cyclohexyl isocyanate or temozolomide treatment. Cell survival was measured by the MTT assay. **$p < .01$. Data shown are means ± SD and are representative of three separate experiments with similar results.

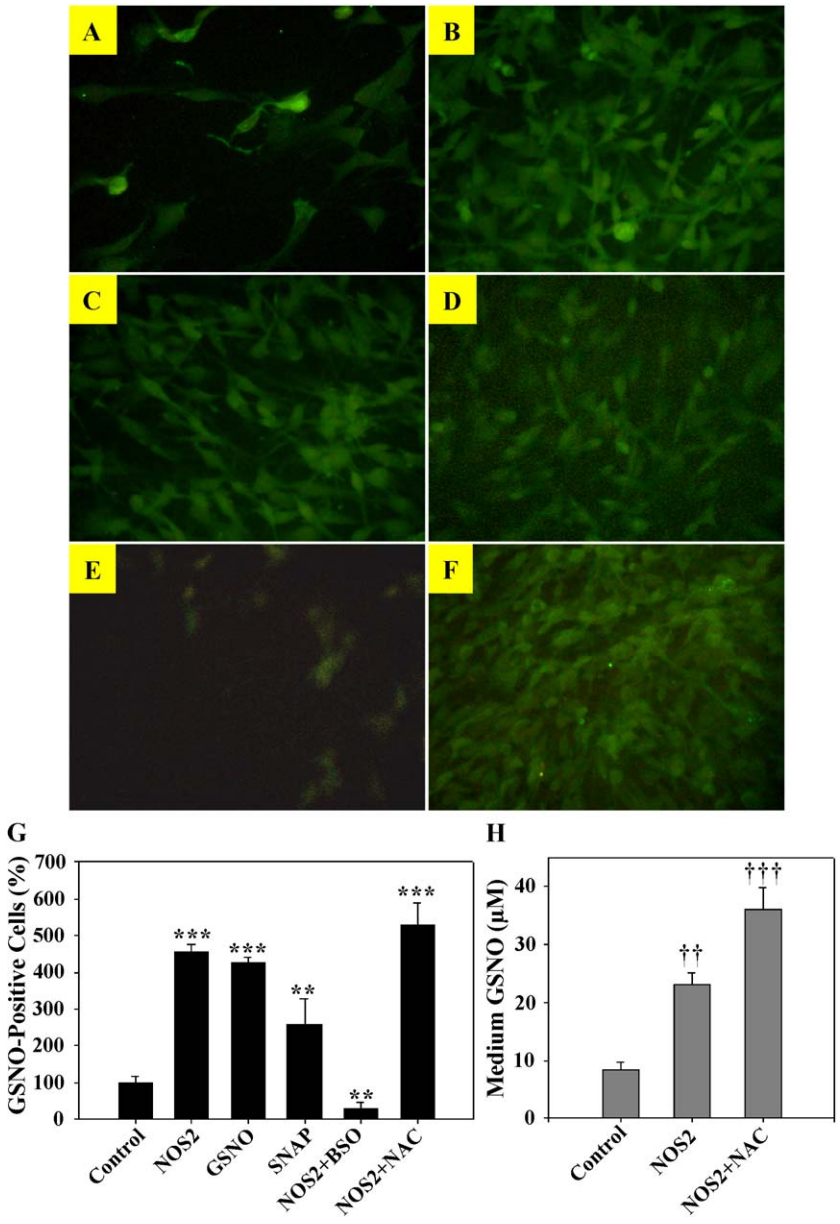

Fig. 3. Altered intracellular GSNO levels and medium GSNO contents in C6 glioma cells under various experimental conditions. (A) Control cells were not treated. (B) Cells were treated with tumor necrosis factor α (TNF-α, 50 ng/ml), interferon-γ (IFN-γ, 200 U/ml), and lipopolysaccharide (LPS, 1 μg/ml) for 24 h to induce NOS2. (C) Cells were incubated with 30 μM GSNO for 24 h. (D) Cells were treated with 130 μM SNAP for 24 h. (E) Cells were treated with 125 μM BSO for 18 h followed by cytokine treatments as described in (B). (F) Cells were exposed to 0.5 mM NAC for 2 h prior to cytokine treatments as described in (B). (A–F) 400 × magnification. (G) Summarized results for the numbers of GSNO-positive cells after various experimental manipulations as described above. Four visual fields were randomly selected for each experimental condition (A–F) to conduct the counting. Means ± SD. $^{**}p < .01$ and $^{***}p < .001$ as compared with untreated control cells. (H) Cells were transfected with NS05 alone for 24 h (*NOS2*) or were previously exposed to 0.5 mM NAC for 2 h before NS05 transfection (*NOS2+NAC*). Cells transfected with pcDNA3.1 served as negative controls (*control*). Medium GSNO was assessed by HPLC as described under Materials and Methods. Data shown are means ± SD. $^{††}p < .01$ and $^{†††}p < .001$ as compared with untreated control cells.

including GSNO (Fig. 3C) and SNAP (Fig. 3D), led to increased numbers of cells positively stained with GSNO antibody as compared with the untreated controls treatments (Fig. 3A). In addition to NO, glutathione is another component that may dictate the formation of GSNO. BSO depletes the cellular glutathione stores, including both the reduced and oxidized forms, by inhibiting γ-glutamylcysteine synthetase, which catalyzes glutathione biosynthesis [25]. NAC is a glutathione precursor that may enrich cellular gluta-

thione stores in glial cells [26]. We therefore pretreated C6 glioma cells with BSO and NAC to respectively deplete and increase cellular glutathione contents before NOS2 expression. The immunofluorescence data clearly revealed that, despite NOS2 expression as confirmed by Griess reaction (data not shown), cells depleted of cellular glutathione stores with BSO preexposure displayed very few cells stained with GSNO antibody (Fig. 3E). In contrast, numerous cells were positive for GSNO immunostaining with glutathione enrichment by NAC on NOS2 overexpression (Fig. 3F). Counting of fluorescent cells in each visual field provided a semiquantitative analysis of the alterations in the numbers of GSNO-positive cells under various experimental manipulations (Fig. 3G).

GSNO is a nitrosylated glutathione. Glutathione can be exported into medium via multidrug resistance protein 1 in cultured rat astroglial cells [27,28]. We therefore speculated that the increased intracellular GSNO contents may indirectly result in heightened medium GSNO levels, which allows HPLC analysis for quantification. Cells were either pretreated with NAC or not pretreated prior to overexpression of NOS2. NAC did not alter NOS2 expression as assessed by the Griess reaction (data not shown). As expected, overexpression of NOS2 alone resulted in the increase in medium GSNO content; glutathione enrichment by NAC treatment (0.5 mM for 2 h) coupled with NOS2 overexpression for an additional 24 h further enhanced medium GSNO level (Fig. 3H). Together, the results shown in Fig. 3 demonstrate that manipulations of NO and/or glutathione content may modulate cellular GSNO levels based on immunofluorescence staining and HPLC analysis.

Effects of glutathione depletion and enrichment on NOS2-induced chemoresistance against carbamoylating agents

Given the hypothesis that GSNO formation is a key step in the development of carbamoylating chemoresistance, various experimental manipulations that modulate GSNO formation should correspondingly affect glioma chemoresistance. We have demonstrated that NOS2 overexpression [18] and exogenous application of GSNO both increased cellular GSNO stores and enhanced chemoresistance (the present study). We further tested the effects of glutathione, another key component for GSNO biosynthesis, on carbamoylating chemoresistance. BSO pretreatment completely abolished NOS2-induced chemoresistance against BCNU (Fig. 4A). Similar effects were observed with other carbamoylating agents including compound 5 (Fig. 4B) and compound 3 (Fig. 4C). Thus, glutathione depletion that has prevented GSNO formation despite overexpression of NOS2 (Fig. 3E) abolishes NOS2-induced chemoresistance against the carbamoylating action of chloroethylnitrosoureas (Fig. 4).

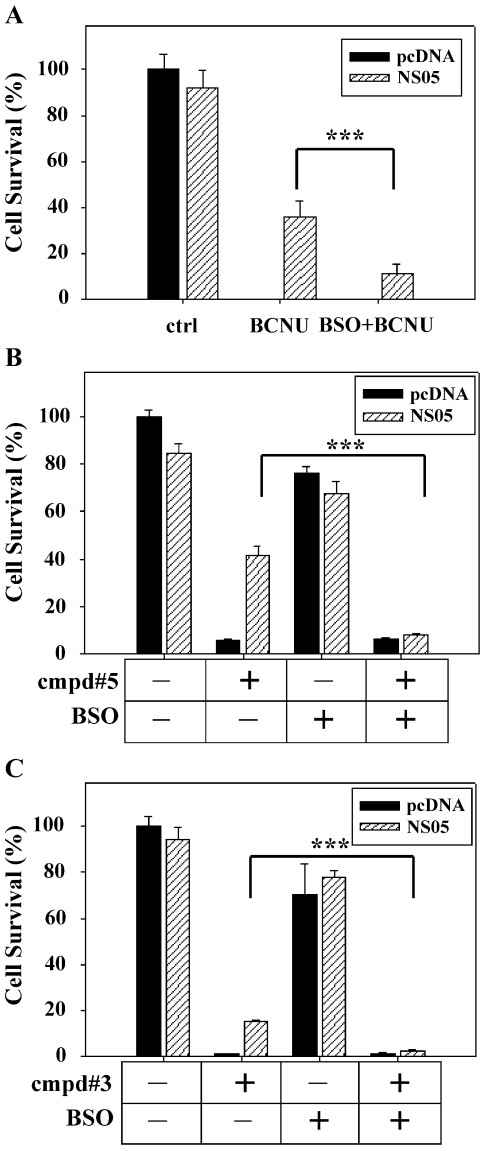

Fig. 4. BSO effects on chemoresistance against carbamoylating agents. (A) Cells transfected with NOS2-expressing construct NS05 (*hatched bars*) or empty vector pcDNA3.1 (*solid bars*) were pretreated with 125 μM BSO alone for 18 h and then exposed to BCNU at 0.47 mM for an additional 12 h (*BSO+BCNU*). Note that BCNU exposure resulted in almost total killing with 0% survival in pcDNA3.1 transfectants with or without BSO pretreatment. Cell survivals are based on the trypan blue exclusion assay. ***$p < .001$. (B, C) Cells transfected with NS05 (*hatched bars*) or pcDNA3.1 (*solid bars*) were pretreated with and without BSO (125 μM for 18 h) before exposure to 0.8 mM compound 5 (B) or 0.6 mM compound 3 (C) for an additional 24 h. ***$p < .001$. Cell survival in (B) and (C) was determined by the MTT assay. Data shown are means ± SD and are representative of three separate experiments with similar results.

We also determined the effect of NAC on NOS2-induced chemoresistance against BCNU and other carbamoylating agents. At concentrations greater than 1

mM, NAC effectively enhanced chemoresistance against BCNU in C6 glioma cells overexpressing NOS2 (Fig. 5A), suggesting a synergistic protective action exerted by NAC and NO, probably via the formation of GSNO. NAC alone was also effective in protecting C6 cells from other carbamoylating agents such as compound 5 (Fig. 5B) and compound 3 (data not shown), but failed to exert similar effects on the alkylating agents including compound 1 and temozolomide (Fig. 5C).

Effects of copper chelators on GSNO- and NOS2-mediated chemoresistance against carbamoylating action

If GSNO plays crucial roles in mediating the observed NOS2 effects against the carbamoylating cytotoxicity of chloroethylnitrosoureas, reagents capable of altering GSNO action are expected to correspondingly modulate the NOS2 action. Copper ions and selective copper chelators have been used to study the biological actions of GSNO [29–37]. We therefore tested the effects of selected copper chelators on GSNO- and NOS2-induced chemoresistance against carbamoylating agents. Neocuproine, a cuprous ion chelator, abolished the chemoresistance exerted by GSNO against compound 5; cuprizone, a cupric ion chelator was, however, without effect (Fig. 6A). Given that neocuproine effectively eliminated GSNO-mediated chemoresistance against compound 5, we predicted that neocuproine should also block NOS2-induced chemoresistance against carbamoylating agents, if GSNO is indeed the mediator of this NOS2 action. As expected, neocuproine, but not cuprizone, dose-dependently reduced chemoresistance against BCNU in C6 glioma cells overexpressing NOS2 (Fig. 6B). That neocuproine was effective in abolishing chemoresistance induced by GSNO as well as that by NOS2 overexpression is consistent with the contention that NOS2-induced chemoresistance against carbamoylating agents is, at least in part, mediated by GSNO.

Effects of other cellular events downstream of NOS2 expression

In addition to a heightened GSNO content, NOS2 expression and subsequent increase in NO synthesis may have other biological actions that could contribute to the observed chemoresistance against carbamoylating activity. A number of other cellular events downstream of NOS2 expression were studied to exclude their potential involvement in conferring chemoresistance. NO may nitrosylate the active cysteine residues on caspases to diminish their activity, leading to an inhibition of apoptotic processes [38]. z-VAD, a broad-spectrum caspase inhibitor, did not confer chemoresistance against BCNU in C6 glioma cells with or without NOS2 overexpression (data not shown). Cyclic GMP (cGMP) is a mediator of

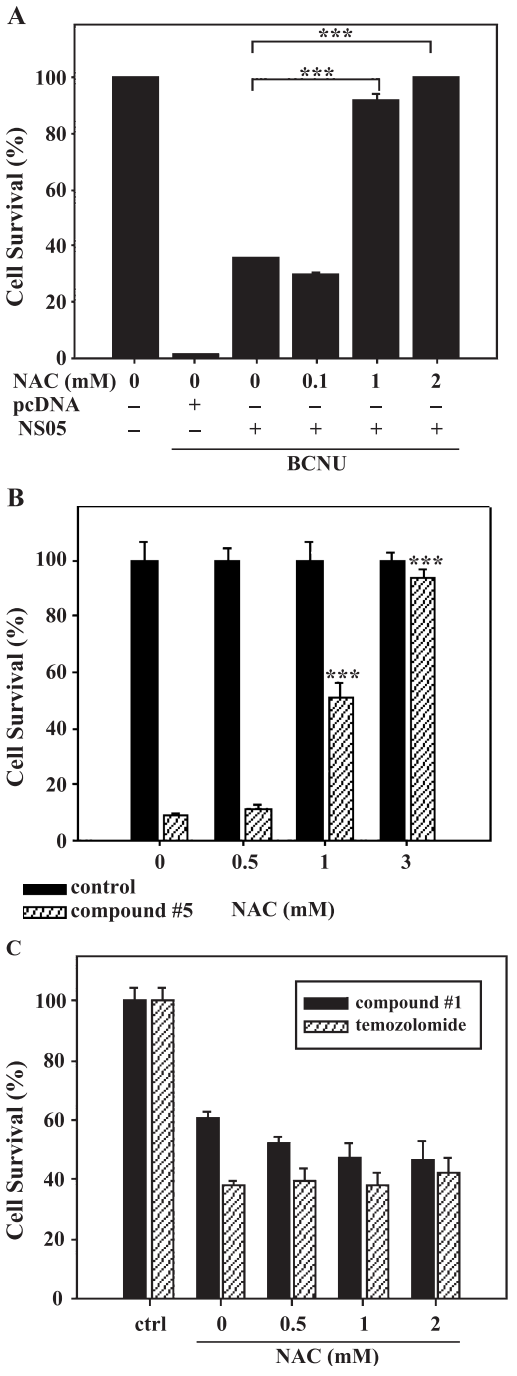

Fig. 5. NAC effects on chemoresistance against carbamoylating and alkylating agents. (A) Cells transfected with NS05 or pcDNA3.1 without or with NAC pretreatment (0.1–2.0 mM for 2 h) followed by BCNU exposure (0.47 mM) for 12 h. ***$p < .001$. (B) C6 glioma cells were pretreated without or with NAC at the indicated concentrations for 2 h before exposure to 0.8 mM compound 5. ***$p < .001$, significant difference from cells treated with compound 5 but without NAC. (C) The experimental protocol was similar to (B) except compound 1 (3 mM) and temozolomide (3 mM) were used. Note that NAC pretreatments did not alter chemoresistance against compound 1 or temozolomide. Cell survival was based on the MTT assay. Data shown are means ± SD and are representative of three separate experiments with similar results.

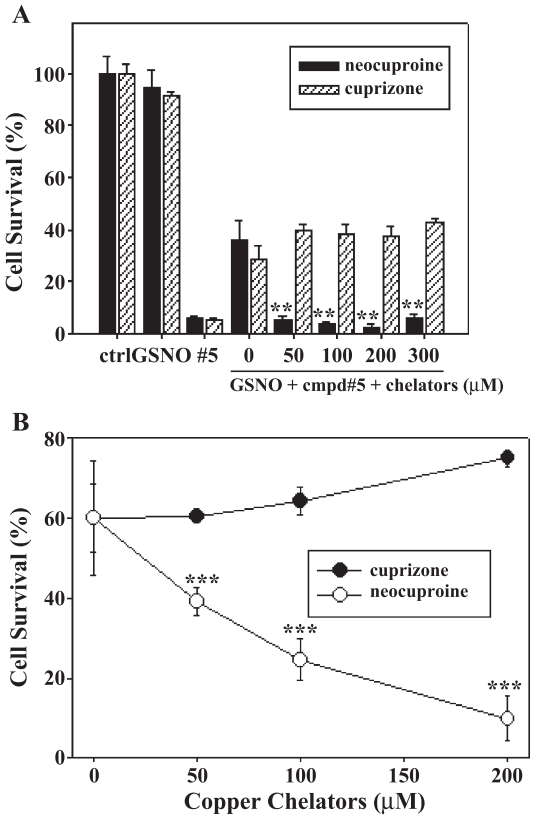

Fig. 6. Effects of copper chelators on GSNO- and NOS2-mediated chemoresistance. (A) Cells were treated with 100 μM GSNO alone (*GSNO*), 0.8 mM compound 5 alone (*#5*), or both reagents without and with neocuproine (*solid bars*) or cuprizone (*hatched bars*) at the indicated concentrations. $^{**}p < .01$ compared with the cells treated with both GSNO and compound 5 but without neocuproine. (B) Cells were transfected with NS05 and then exposed to BCNU (0.47 mM) for 12 h without and with neocuproine (*open circles*) or cuprizone (*solid circles*) at the indicated concentrations. $^{***}p < 0.001$ compared to cells treated with the same concentrations of cuprizone. Cell survival rates were based on the MTT assay. Data shown are means ± SD and are representative of three experiments with similar results.

selected NO actions in primary astrocyte cultures [39]. 8-Bromo-cGMP, a slowly hydrolyzable analog of cGMP that activates its downstream kinases, did not enhance chemoresistance against BCNU in C6 glioma cells (data not shown). Finally, neither exogenous sodium nitrite nor conditioned medium derived from cells overexpressing NOS2 with elevated nitrite/nitrate levels conferred chemoresistance against BCNU (data not shown).

DISCUSSION

We have reported previously that overexpression of NOS2 confers chemoresistance against carbamoylating agents including BCNU, CCNU, compound 5, compound 3, 2-chloroethyl isocyanate, and cyclohexyl isocyanate in C6 glioma cells [18]. In the present study, we further characterize the molecular mechanism underlying this novel in vitro effect. Our findings suggest that GSNO likely plays an important role in this NOS2-induced chemoresistance against carbamoylating agents. Several lines of evidence support this contention. First, among the three NO donors tested, only GSNO conferred BCNU chemoresistance (Fig. 1). Exogenous GSNO also enhanced chemoresistance against all the carbamoylating agents tested including compound 5, compound 3, cyclohexyl isocyanate, and 2-chloroethyl isocyanate, but not against alkylating agents such as compound 1 and temozolomide (Fig. 2). Second, experimental manipulations expected to increase or decrease cellular GSNO content (Fig. 3) correspondingly affected carbamoylating chemoresistance (Figs. 4 and 5) [18]. Third, copper ions have been shown to modulate GSNO action; selective chelators for Cu^{+} and Cu^{2+} were used to characterize the copper-mediated GSNO metabolism [29,31]. We found that neocuproine (a Cu^{+} chelator), but not cuprizone (a Cu^{2+} chelator), was effective in blocking the development of chemoresistance against carbamoylating agents induced by NOS2 overexpression as well as that induced by exogenous GSNO (Fig. 6). That neocuproine, which was capable of abolishing GSNO-dependent chemoresistance, effectively neutralized chemoresistance associated with NOS2 further strengthens the notion that NOS2-induced chemoresistance against carbamoylating agents depends on the formation of GSNO. Finally, other cellular events downstream of NO were excluded as being responsible for NOS2-induced chemoresistance against carbamoylating agents. These include NO-dependent inhibition of caspase activity, activation of guanylyl cyclase with resultant activation of cGMP-dependent protein kinases, and an increase in the nitrite/nitrate content (data not shown).

GSNO has been implicated in various biological functions that are mostly attributed to its ability to release NO. Degradation of GSNO with resultant release of NO and GSSG can be catalyzed by Cu^{+} [24,30]. Recently, GSNO has also been reported to act as an antioxidant two orders of magnitude more potent than GSH, capable of protecting brain dopaminergic neurons against iron-induced oxidative stress [40,41]. However, decomposed GSNO lacks such antioxidative and neuroprotective activities (Yang and Hsu, unpublished observations). It is possible that NO slowly released from GSNO is itself an antioxidant [42]. The redox versatility of NO may allow its interconversion from a cytotoxic to an antioxidative, and hence a cytoprotective, species by changes in the ambient redox milieu [43]. Further, the biological half-life of NO as a free radical lasts only seconds but can be prolonged to hours after its conversion to GSNO, which may then release NO at a slower and steadier pace to exert antioxidant activity [14]. Consistent with this notion is the observation that the release of NO radical from GSNO has recently been shown, based on electron

paramagnetic resonance spectroscopy studies, to be a dynamic process with continuous release and trapping of NO molecules, thereby substantially extending the biological half-life of available NO [44]. As compared with O_2, NO is also relatively inert to reduction to nitroxyl anions under physiological conditions [45]. Wink et al. [46] have compared the effect of various NO donors on hydrogen peroxide-mediated toxicity and established a direct correlation between NO formation and cytoprotection. Specifically they showed that SIN-1 (a peroxynitrite-releasing agent) and sodium nitroprusside (SNP), both of which do not release appreciable amounts of NO, enhanced hydrogen peroxide-mediated cytotoxicity, whereas GSNO, SNAP, and DEA/NO, which release NO, exerted protective effects in V79 Chinese hamster lung fibroblasts [46]. In addition, only those NO donors with longer half-lives protect rat oligodendrocyte cultures from intracellular glutathione depletion caused by cysteine deprivation [47]. We have observed similar findings that SIN-1 failed to exert any protective effects but GSNO and SNAP pretreatment as well as NOS2 overexpression were effective in protecting C6 glioma cells against BCNU carbamoylating cytotoxicity. Cellular proteins undergoing carbamoylation may lose their biological functions. Carbamoylation may render those enzymes critically involved in maintaining cellular redox homeostasis, such as glutathione reductase, irreversibly nonfunctional, thereby leading to accumulation of GSSG [48–50]. In this respect carbamoylation may be considered as a chemical-induced oxidative stress that can be antagonized by antioxidants, namely, GSNO and NAC, as shown in the present study. The beneficial action of GSNO has also been revealed in an in vivo paradigm, in which GSNO given before reperfusion afforded tissue protection against postischemic myocardial injury in rabbits [51]. These results together demonstrate a cytoprotective role of GSNO, which may be caused by the sustained release of NO itself in various experimental settings both in vitro and in vivo. Overall, results from the present study are compatible with the contention that GSNO may also serve as a NO reservoir that slowly releases NO to antagonize oxidative stress induced by exposure to carbamoylating chemotherapeutic agents.

An increase in the number of cells immunostained with GSNO-specific antibody in C6 glioma cells was noted after exposure to 300 μM Angeli's salt for 12 h, which also resulted in cytoprotective effects against 100 μg/ml BCNU (data not shown). Angeli's salt is known to release nitroxyl anion. Under appropriate conditions, the nitroxyl anion released from Angeli's salt may be oxidized into NO radical by one-electron oxidants including ferricyanide ($Fe(III)(CN_6)^{3-}$), which confers dramatic protection against Angeli's salt-mediated cytotoxicity in Chinese hamster V79 lung fibroblast cells [52]. Furthermore, copper ions [53] and cytochrome P450 have been suggested to play a role in converting the nitroxyl anion generated by Angeli's salt into NO, which was implicated in Angeli's salt-mediated vasodilatory actions on rat aorta [54]. In contrast, NO is relatively inert to reduction to nitroxyl anions under physiological conditions [45]. Thus, the interesting possibility of GSNO formation by interaction of glutathione with NO indirectly produced from Angeli's salt, possibly due to the presence of one-electron oxidant metal ions in culture medium, cannot be excluded. However, nitroxyl anions released by Angeli's salt have also been implicated in injuries caused by myocardial ischemia/reperfusion in vivo [51] and lung fibroblast cells in vitro [52]. The detailed mechanisms underlying these observations regarding the effects of Angeli's salt require extensive further investigation and are beyond the scope of the present study.

While causing chemoresistance at concentrations below 300 μM, GSNO over 400 μM began to cause cytotoxicity in C6 cells independent of BCNU exposure (data not shown). GSNO at 1 mM has been shown to substantially inhibit mitochondrial complex I activity [55]. GSNO may be decomposed into NO and GSSG, both of which act as pro-oxidants at higher concentrations. Thus, the concentrations of this endogenous and physiologically relevant NO carrier in tumor cells appear to be critical in determining whether it causes carbamoylating chemoresistance or cytotoxicity. Further investigation is required to examine whether GSNO metabolism in malignant brain tumors is distinct from that in normal brain tissues or meningiomas. *S*-Nitrosocysteinylglycine (CGSNO) is a potential metabolic intermediate of GSNO recently proposed as a novel cGMP-independent mechanism for regulating certain biological effects of GSNO [56–58]. GSNO has been previously demonstrated to be a substrate for γ-glutamyl transpeptidase (γ-GT), an enzyme hydrolyzing the γ-glutamyl moiety of glutathione to form glutamate and cysteinylglycine. CGSNO is more susceptible to transition metal ion-dependent decomposition than GSNO [59]. In the present study, we demonstrated the pivotal role of GSNO formation mediating NOS2-dependent chemoresistance against carbamoylating agents including BCNU. Whether GSNO is degraded into CGSNO by γ-GT prior to release of NO in our experimental setting and the potential roles of CGSNO in carbamoylating chemoresistance require further studies.

Chloroethylnitrosoureas, especially BCNU, have been a mainstay in the adjunct chemotherapy of GBM following surgical resection and radiation. Unfortunately, the clinical outcomes with the combination of these three modalities of treatment remain far from satisfactory. The underlying mechanisms of chemoresistance against chloroethylnitrosoureas such as BCNU are not fully

understood. The present study reveals that GSNO derived from NO and glutathione may directly alter glioma resistance to carbamoylating chemotherapeutic agents. Heightened NOS2 expression has been detected in malignant glioblastomas as compared with normal brain tissues [12] or meningiomas [13]. Surgical procedures or radiation therapy may also result in inflammatory responses leading to enhanced NOS2 expression. Formation of GSNO may therefore occur in malignant brain tumors prior to the initiation of BCNU therapy, thereby causing chemoresistance against carbamoylating agents. Interestingly, we have observed that SNAP, a NO-releasing nitrosothiol, was effective in increasing GSNO formation (Fig. 3D) and neutralizing BCNU cytotoxicity under a pretreatment condition (data not shown), but not in a cotreatment paradigm (Fig. 1B). Similarly, overexpression of NOS2 also has to occur before application of BCNU to develop NO-mediated chemoresistance [18]. It is likely that SNAP, a nitrosothiol, is capable of increasing the cellular GSNO content as NOS2 and GSNO do, but it takes additional time for GSNO to be synthesized. In contrast, GSNO is expected to exert its anticarbamoylation effects on application without intermediate synthetic steps.

In conclusion, results from this study suggest that GSNO formation as a result of NOS2 expression may represent an important mechanism underlying the development of chemoresistance against carbamoylating agents in glioma cells that is independent of the well-known vascular actions of NO including angiogenesis [60]. Such an effect is also distinct from other known mechanisms of resistance to chemotherapeutic agents, such as the induction of O6-alkylguanine-DNA alkyltransferase [61] and DNA mismatch repair [62], which are more likely to render GBM resistant to the alkylating action of chloroethylnitrosoureas. Application of NOS inhibitors to reduce NO formation is within reach in clinical studies [63]. Pharmacological modulation of NO or GSNO formation may open a novel avenue to reduce chemoresistance against BCNU in GBM.

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ABBREVIATIONS

BCNU — 1,3-bis(2-chloroethyl)-1-nitrosourea
BSO — buthionine sulfoximine
CCNU — 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea
DEA/NO — 2-(*N,N*-diethylamino)-diazenolate-2-oxide
GBM — glioblastoma multiforme
GSH — reduced form of glutathione
GSNO — *S*-nitrosoglutathione
GSSG — oxidized form of glutathione
IFN-γ — interferon-γ
LPS — lipopolysaccharide
MTT — 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
NAC — *N*-acetyl-L-cysteine
SIN-1 — 3-morpholinosydnonimine
SNAP — *S*-nitroso-*N*-acetyl-DL-penicillamine
SNP — sodium nitroprusside
TNF-α — tumor necrosis factor α