Induction of mild intracellular redox imbalance inhibits proliferation of CaCo-2 cells

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ABSTRACT **Intracellular redox status plays a critical role in cell function, such as proliferation. Oxidative stress, which elicits redox imbalance, also affects cell growth. Therefore, it is often difficult to distinguish the effects of redox imbalance from those of oxidative stress. The objective of this study was to determine the role of redox imbalance independent of reactive oxygen species (ROS) production, in proliferation of human colonic CaCo-2 cells. Low concentrations of diamide plus 1,3-bis(2 chloroethyl)-1-nitrosourea (BCNU) increased intracellular GSSG and decreased GSH and the GSH:GSSG ratio. These changes occurred within 30 min, which preceded a decrease in thymidine incorporation at 6 and 24 h. ROS formation was not detected under these conditions. This suppression of cell proliferative activity was attenuated by N-acetyl cysteine, in parallel with restoration of the intracellular GSH redox status. DL-buthionine-[S, R]-sulfoximine (BSO) decreased intracellular GSH level, but did not change the GSH:GSSG ratio. BSO alone had no effect on cell proliferation, but its presence exaggerated the suppressive effect of diamide plus BCNU. Flow cytometric analysis showed that cells were arrested at G_1-to-S transition and G_2/M phase. Collectively, this study shows that mild intracellular redox imbalance inhibited cell proliferation independent of ROS generation. Moreover, cells with compromised cellular GSH were susceptible to redox imbalance-induced inhibition of proliferation.—Noda, T., Iwakiri, R., Fujimoto, K., Aw, T. Y. Induction of mild intracellular redox imbalance inhibits proliferation of CaCo-2 cells. *FASEB J.* 15, 2131–2139 (2001)**

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IN RECENT YEARS, there is increasing recognition that intracellular redox status affects cellular and molecular events in various cells. Redox status modulates protein activities (1), the ability of certain transcriptional factors to bind to their cognate DNA (2), signal transduction (3, 4), cell necrosis (5), cell apoptosis (5, 6), and cell proliferation (7, 8).

Oxidative stress is an important pathophysiological stimulus that affects the cellular redox status. However, besides inducing redox shift, oxidative stress can directly affect cell functions such as enzyme activities, cell signaling, and cellular responses (9). Often the effects of redox changes overlap with those of oxidative stress. For that reason, it is sometimes unclear whether cellular responses elicited by oxidative stress are mediated by reactive oxygen species (ROS) directly or by ROS-induced redox imbalance.

Glutathione (L-γ-glutamyl-L-cysteinylglycine) is a ubiquitous intracellular thiol present in all tissues. Its reduced form (GSH) and oxidized form (glutathione disulfide, GSSG) constitute the major thiol redox system in cells, and the redox status of GSH and GSSG is of crucial importance for cellular function (1). Glutathione has a role in signal transduction (3, 4, 10), gene expression (11), modulation of protein function (3, 12), necrosis (5), and apoptosis (13, 6). It is also associated with cell proliferation (14–19) and affects growth factor functions (20, 21). However, the role of thiol-disulfide balance in cell proliferation is unresolved.

In the current study, we used human colon cancer CaCo-2 cells to test the hypothesis that cell proliferation is responsive to the cellular GSH/GSSG status. The specific objectives were to determine *1)* whether the induction of mild GSH/GSSG imbalance affects cell proliferation independent of ROS and *2)* redox-induced cell cycle change, and *3)* whether redox change underlies the stimulatory effect of EGF on cell proliferation.

MATERIALS AND METHODS

Reagents

Diamide, N-acetyl-L-cysteine (NAC), DL-buthionine-[S, R]-sulfoximine (BSO), RNAase, and rhodamine 123 were obtained from Sigma Chemical (St. Louis, MO). 1,3-bis (2 chloroethyl)-1 nitrosourea (BCNU) was purchased from Bristol-Myers (Princeton, NJ). Dihydro rhodamine 123 (DHR) was obtained from Molecular Probes (Eugene, OR). Epidermal growth factor (EGF) was from GIBCO-BRL (Grand Island, NY). [Methyl-^{3}H]-thymidine and L-[1-^{14}C] ornithine were obtained from NEN Products (Boston, MA). Fetal bovine serum was obtained from Atlanta Biologicals (Norcross, GA). Dulbecco's modified Eagle

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medium (DMEM) and other cell culture supplies were purchased from GIBCO-BRL. All other chemicals were of reagent grade and were obtained from local sources.

Cell culture and treatment

The human colon cancer cell line CaCo-2 was obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in 75 cm^2 culture flasks in DMEM supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 100 U/ml penicillin, and 10 mg/ml gentamicin. The cell cultures were incubated in humidified atmosphere with 5% CO_2/95% air at 37°C. The culture medium was changed every 2 days.

For experimentation, CaCo-2 cells were seeded in 6- or 24-well plates and cultured to 60% confluence in DMEM with serum. Before incubation with redox agents, cell culture media were replaced with fresh serum containing DMEM. In some experiments, incubations were performed in serum-free DMEM. Whenever present, reagents were added to cell cultures at the following final concentrations: diamide, 30 μM; BCNU, 30 μM; NAC, 5 mM; EGF, 100 ng/ml. BCNU and NAC were administered 30 min before diamide treatment. Some cultures were pretreated with 5 mM BSO for 12 h to lower the baseline intracellular glutathione concentration before treatment with diamide or diamide plus BCNU. In addition, BSO was also present throughout the incubation period to prevent the resynthesis of glutathione.

GSH and GSSG determination

Intracellular GSH and GSSG levels were determined by the high-performance liquid chromatography method of Reed et al. (22). Cells were treated with ice-cold 5% trichloroacetic acid (TCA), followed by centrifugation to remove TCA-insoluble proteins. The acid supernatant was derivatized with 6 mM iodoacetic acid and 1% 2, 4-dinitrofluorobenzene to yield the S-carboxylmethyl and 2, 4-dinitrophenyl derivatives of GSH and GSSG. Separation of GSH and GSSG derivatives was achieved on a 25 cm × 4.6 mm × 10 μm Lichrosorb NH_2 column.

[^{3}H-] thymidine incorporation

[^{3}H-] Thymidine (1 μCi) was added to each well 3 h before collecting cells. At each time point indicated, labeled monolayer was washed twice with phosphate-buffered saline (PBS) and the cells were detached with trypsin-EDTA. Cells were precipitated with 5% TCA at 4°C. The precipitate was washed twice with 95% ethanol at 4°C, followed by drying at room temperature, and solubilized with 1 N NaOH. Aliquots of dissolved precipitate were mixed with Poly-fluor (Packard, Meriden, CT) and the cell-associated radioactivities were determined using a liquid scintillation counter.

Ornithine decarboxylase (ODC) activity assay

At each time point, the cells were detached with trypsin-EDTA. After washing twice with PBS, cells were collected by centrifugation. ODC activity was assayed by a radiometric technique (23). Briefly, cells were resuspended in 500 μl of 0.1 M Tris buffer (pH 7.4) containing 1 mM EDTA, 50 μM pyridoxal 5-phosphate, and 5 mM dithiothreitol and homogenized. After centrifugation at 12,000 rpm for 30 min, 200 μl aliquot of the supernatant was incubated in stoppered vials in the presence of 3.5 nmol of L-[1-^{14}C] ornithine (50 mCi/mmol) for 1 h at 37°C. The $^{14}CO_2$ liberated by the decarboxylation of ornithine was trapped on filter paper impregnated with 20 μl of 2 N NaOH, which was suspended above the reaction mixture. The reaction was stopped by the addition of 0.3 ml of 10% TCA. The filter paper was placed in Poly-fluor and radioactivities of the $^{14}CO_2$ were measured using a liquid scintillation counter. Results are expressed as picomoles CO_2 per milligram protein per hour.

Cell cycle analysis by flow cytometry

At the end of each period, the cells were trypsinized, washed with cold PBS, and fixed in 70% ethanol for 30 min at 4°C. Fixed cells were washed with PBS and incubated in 1 mg/ml DNAase-free RNAase for 40 min at 37°C. The RNAase was preheated to 100°C for 15 min to inactivate DNAase. Propidium iodide (PI, 50 μg/ml) was added and samples were incubated at 4°C for 30 min in the dark. Flow cytometric analysis was performed using a FACS Vantage flow cytometer (Becton Dickinson, San Jose, CA). In each experiment, a minimum of 5×10^5 cells per sample was analyzed. Data were analyzed using Cell Quest software (Becton Dickinson, San Jose, CA) and gated on pulse-processed PI signals to exclude doublets and large aggregates, using a multiparameter gate strategy.

Determination of cellular oxidant production

Oxidant formation was measured using the oxidant-sensitive nonfluorescent probe DHR. Previous studies have used DHR to detect reactive oxygen species in cells (24). Because the intracellular oxidation of DHR is mediated by biological oxidants like peroxynitrite and a variety of secondary hydrogen peroxide-dependent intracellular reactions that includes H_2O_2-cytochrome *c* and H_2O_2-Fe (2-) (24, 25), detection of increased oxidation of this probe is a useful marker of a change in general cellular oxidant production. In addition, the fluorometric determination of rhodamine from DHR oxidation has been used to determine the interactions between superoxide and nitric oxide (26). Intracellularly, DHR is oxidized by two electrons that results in the formation of rhodamine 123, which possesses high molar absorptivity and is fluorescent. Since excitation of rhodamine at 500 nm results in light emission at 536 nm, measurement of the amount of rhodamine at these excitation/emission wavelengths provides a reasonable quantification of oxidant production.

DHR was prepared as a 25 mM stock solution in nitrogen-purged dimethyl formamide (DMF) and stored in the dark at −20°C. On the day of each experiment, stock DHR was diluted afresh with DMF and added to cells at a final concentration of 5 μM. Cells were then exposed to diamide or diamide plus BCNU for 6 or 24 h. At each time, cells were washed twice with PBS. The cells were harvested using a scraper in 2 ml of PBS and sonicated using a Braun-Sonic sonicator (B. Braun Biotech Int., Allentown, PA). Rhodamine123 accumulation was quantified using an AMINCO Bowman Series 2 luminescence spectrophotometer (Thermo Spectronic, Rochester, NY) at excitation and emission wavelengths of 500 nm and 536 nm, respectively. Results were expressed as relative fluorescence unit/mg protein.

Cell count and viability

Cells were counted using a hemocytometer in the presence of 0.5% trypan blue. The changes in cell number after treatments with redox agents are presented as fold change above the starting cell number. Cell viability was determined as the percentage of cells that excluded trypan blue.

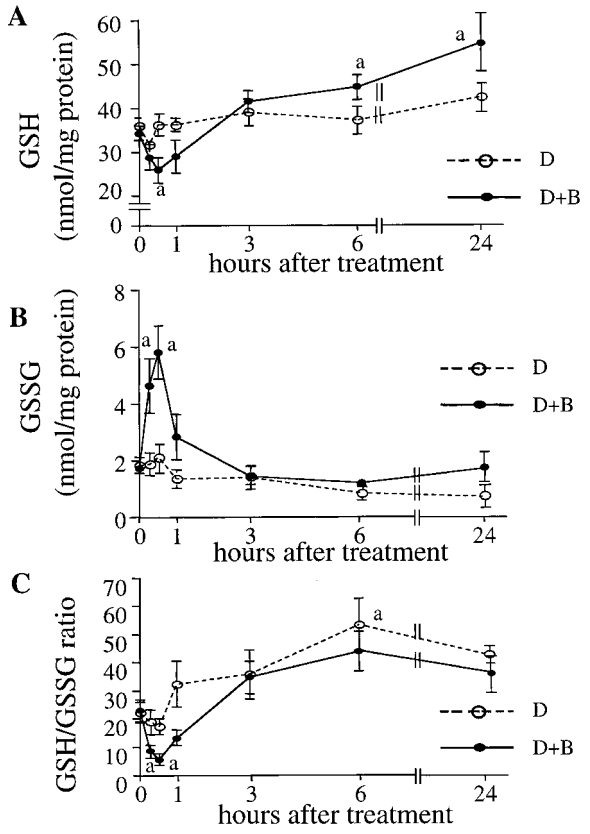

Figure 1. Kinetic changes of intracellular GSH/GSSG status induced by 30 μM diamide (D: dotted line) or 30 μM diamide plus 30 μM BCNU (D+B: solid line) in CaCo-2 cells. Cells were cultured in serum-containing DMEM and treated with diamide. BCNU was added 30 min before diamide treatment. *A)* GSH; *B)* GSSG; *C)* GSH:GSSG ratio. Results are the mean ± SE of 4 separate experiments. a: $P < 0.05$ vs. each respective value at 0 h.

Protein assay

Protein was measured using Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's protocol.

Statistical analysis

Results are expressed as mean ± SE. Data were analyzed using a one-way ANOVA with Bonferroni corrections for multiple comparisons. *P* values of < 0.05 were considered statistically significant.

RESULTS

Kinetic changes of intracellular redox status induced by low-dose diamide plus BCNU

Diamide is a cell-permeant thiol agent that oxidizes GSH to GSSG nonenzymatically independent of oxyradical generation (27); BCNU is an inhibitor of glutathione reductase that blocks the conversion of GSSG to GSH (28). **Figure 1** illustrates the kinetics of intracellular GSH, GSSG, and the GSH:GSSG ratio in cells exposed to 30 μM diamide or 30 μM diamide plus 30 μM BCNU. Treatment of cells with 30 μM diamide alone did not cause a significant change in intracellular GSH, GSSG, or the GSH:GSSG ratio, indicating that this concentration of diamide was insufficient to affect the overall cellular glutathione status under steady state conditions. In addition, cellular GSH/GSSG status was unchanged in cells treated with 30 μM BCNU alone (data not shown). On the other hand, 30 μM diamide plus 30 μM BCNU decreased GSH and elevated GSSG levels within 30 min, resulting in a marked decrease in the GSH:GSSG ratio. This redox imbalance caused by diamide plus BCNU returned to baseline levels within 3 h. The intracellular GSH levels at 6 and 24 h after treatment with diamide plus BCNU were significantly elevated above the pretreatment value (Fig. 1*A*), but these increases in cellular GSH did not alter the GSH:GSSG ratio (Fig. 1*C*).

Based on these results, subsequent experiments were performed using 30 μM diamide plus 30 μM BCNU treatment to induce mild redox imbalance and to examine its influence on cell proliferation.

Effect of low-dose diamide plus BCNU treatment on cell proliferation

The effects of 30 μM diamide plus 30 μM BCNU on proliferation of CaCo-2 cells incubated in serum-containing DMEM are summarized in **Fig. 2**. ^{3}H-Thymidine incorporation in cells treated with diamide plus BCNU

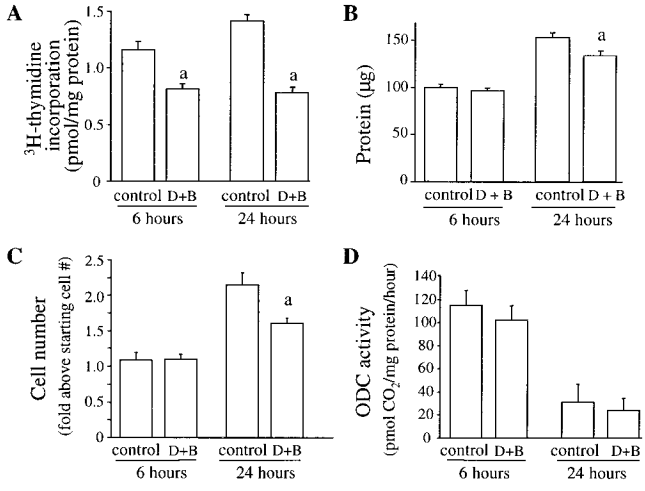

Figure 2. Effect of 30 μM diamide plus 30 μM BCNU on cell proliferation in CaCo-2 cells under serum-replete conditions. Cells were cultured in serum-containing DMEM and incubated with diamide plus BCNU for 6 or 24 h. BCNU was added 30 min before diamide treatment. Control: no treatment, D+B: 30 μM diamide plus 30 μM BCNU. *A)* ^{3}H-thymidine incorporation; *B)* total protein; *C)* cell number expressed as fold change above the starting cell number; *D)* ODC activity. Results are the mean ± SE of 5 separate experiments. a: $P < 0.05$ vs. each corresponding control.

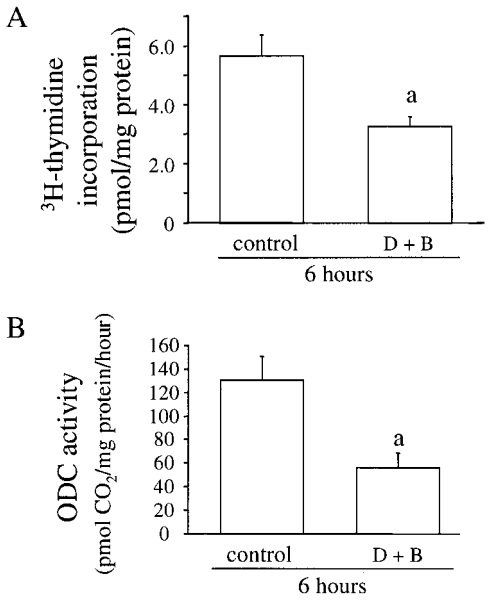

Figure 3. Effect of 30 μM diamide plus 30 μM BCNU on cell proliferation in CaCo-2 cells under serum-free conditions. Cells were cultured in serum-free DMEM for 3 days and incubated with diamide plus BCNU for 6 h. BCNU was added 30 min before diamide treatment. Control: no treatment, D+B: 30 μM diamide plus 30 μM BCNU. *A)* ^{3}H-thymidine incorporation; *B)* ODC activity. Results are the mean ± SE of 5 separate experiments. a: $P < 0.05$ vs. each corresponding control.

was significantly lower than that in untreated cells at 6 and 24 h (Fig. 2*A*). Notably, total protein levels (Fig. 2*B*) and cell numbers (Fig. 2*C*) in untreated cells and cells treated with diamide plus BCNU were increased at 24 h compared with the values at 6 h. At 6 h, there was no difference in protein content or cell number between treated and untreated cells. However at 24 h, these two parameters were significantly lower in cells treated with the redox agents. Treatment of cells with 30 μM diamide or 30 μM BCNU separately had no effect on ^{3}H-thymidine incorporation (data not shown). Together, the decrease in thymidine incorporation, protein content, and cell number induced by 30 μM diamide plus 30 μM BCNU is consistent with a suppression of cell proliferative activity, which paralleled an early disruption in the cellular redox status. Diamide plus BCNU did not affect ODC activity, a common biochemical marker of intestinal proliferation, but overall ODC activity was significantly lower at 24 h than at 6 h (Fig. 2*D*).

Figure 3 shows the effects of 30 μM diamide plus 30 μM BCNU on proliferation of CaCo-2 cells incubated in serum-free DMEM. At 6 h, ^{3}H-thymidine incorporation in cells treated with diamide plus BCNU was significantly lower than that in untreated cells (Fig. 3*A*), which were similar to the observations in cells cultured in serum-containing media (see Fig. 2*A*). However, ODC activity in cells treated with diamide plus BCNU was suppressed at 6 h compared with untreated cells (Fig. 3*B*).

Modulation of GSH/GSSG status and cell proliferation by NAC

L-NAC is an important precursor of GSH. It is a thiol-containing compound with reducing activity (29). NAC (5 mM) abolished the decrease in intracellular GSH and the increase in intracellular GSSG induced by 30 μM diamide plus 30 μM BCNU at 30 min after treatment (**Fig. 4*A, B***, respectively). Accordingly, the administration of NAC resulted in an elevated GSH: GSSG ratio in cells treated with diamide plus BCNU (Fig. 4*C*). In addition, NAC inhibited the suppression of ^{3}H-thymidine incorporation in cells treated with diamide plus BCNU at 6 h and reversed the redox-induced decrease in cell number at 24 h (Fig. 4*D*). These results indicate that the disruption of normal cellular GSH/GSSG status was responsible for the inhibition of proliferation of cells exposed to diamide plus BCNU.

Relationship between intracellular GSH-GSSG status and cell proliferation

BSO is a potent inhibitor of γ-glutamyl cysteine synthetase, the rate-limiting step in GSH synthesis (30). BSO (5 mM) markedly decreased intracellular GSH at 30 min, which declined further at 24 h (**Fig. 5*A***). Notably, intracellular GSH levels in cells treated with BSO was significantly lower than the levels achieved after treatment with 30 μM diamide plus 30 μM BCNU (Fig. 5*A*). The combined treatment with BSO and

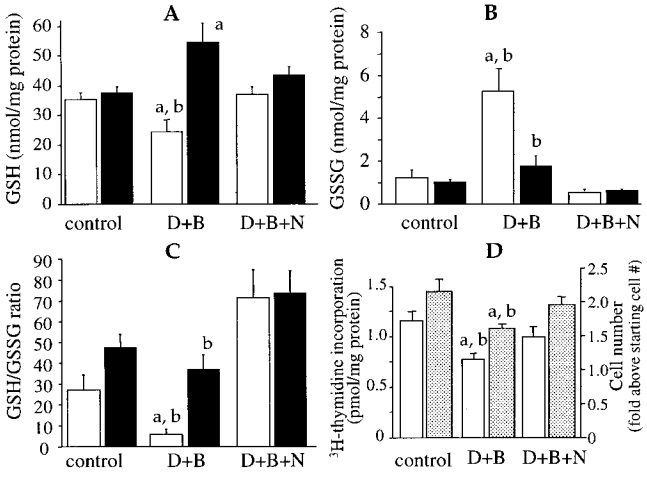

Figure 4. Modulation of GSH/GSSG status and cell proliferation by NAC in CaCo-2 cells. Cells were cultured in serum-containing DMEM and incubated with 30 μM diamide plus 30 μM BCNU with or without 5 mM NAC. BCNU and NAC were administered 30 min before diamide treatment. Control: no treatment, D+B: 30 μM diamide plus 30 μM BCNU, D+B+N: 30 μM diamide plus 30 μM BCNU with 5 mM NAC. *A–C)* GSH, GSSG, and GSH:GSSG ratio at 30 min (open bar) or 24 h (filled bar), respectively. *D)* Data for ^{3}H-thymidine incorporation at 6 h (open bar) and cell number at 24 h (dotted bar). Results are the mean ± SE of 5 separate experiments. a: $P < 0.05$ vs. each corresponding control. b: $P < 0.05$ vs. each corresponding D+B+N.

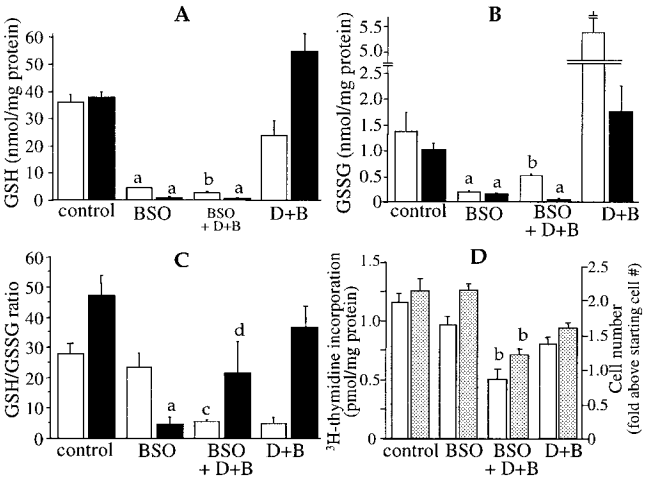

Figure 5. Relationship between intracellular GSH-GSSG status and cell proliferation in CaCo-2 cells. Cells were cultured in serum-containing DMEM and incubated with or without 30 μM diamide plus 30 μM BCNU. BCNU was added 30 min before diamide treatment. BSO was administered 12 h before diamide treatment and was present throughout the incubation period. Control: no treatment, BSO; pretreatment of 5 mM BSO alone, BSO+D+B: 30 μM diamide plus 30 μM BCNU after pretreatment of 5 mM BSO, D+B: 30 μM diamide plus 30 μM BCNU. *A–C)* GSH, GSSG, and GSH: GSSG ratio at 30 min (open bar) or 24 h (filled bar), respectively. *D)* Data for ^{3}H-thymidine incorporation at 6 h (open bar) and cell number at 24 h (dotted bar). Results are the mean ± SE of 5 separate experiments. a: $P < 0.05$ vs. control and D+B, b: $P < 0.05$ vs. control, BSO, and D+B, c: $P < 0.05$ vs. control and BSO, d: $P < 0.05$ vs. control, respectively at corresponding time points.

diamide plus BCNU caused a more severe decrease in GSH level than BSO treatment alone at 30 min (Fig. 5*A*). Figure 5*B* shows that BSO also diminished intracellular GSSG, which is consistent with an overall decrease in total cellular glutathione levels after BSO treatment of cells. However, exposure of BSO-treated cells to diamide plus BCNU resulted in a significant increase in GSSG at 30 min, indicating a potentiation of oxidative stress under these conditions of severely compromised cellular GSH status. The degree of GSSG increase after BSO treatment (without or with diamide plus BCNU) was significantly lower than the GSSG values in cells without BSO treatment (Fig. 5*B*). Although BSO treatment alone did not change GSH: GSSG ratio at 30 min, the added stress of diamide plus BCNU resulted in a significant decrease in the GSH: GSSG ratio to the same extent as that seen with diamide plus BCNU treatment without BSO pretreatment (Fig. 5*C*). However, under these two conditions, the GSH/GSSG redox status returned to near control values by 24 h. ^{3}H-Thymidine incorporation and changes in cell number after BSO treatment was unaltered compared with control (Fig. 5*D*). However, exposure of BSO-treated cells to diamide plus BCNU caused a significant decrease in cell number and in ^{3}H-thymidine incorporation compared with control, treatment with BSO alone, and treatment with diamide plus BCNU (Fig. 5*D*). Together, these results indicate that a mere compromise in the cellular GSH pool per se has minimal influence on cell proliferative activity. Rather, the data support the notion that an early and/or abrupt change in the GSH:GSSG ratio shortly (e.g., 30 min) after oxidative challenge, rather than gradual or later changes (e.g., 24 h) in redox status, is the important determinant in the suppression of cell proliferation. Even though the decrease in intracellular GSH level per se did not inhibit cell proliferation, it enhanced the susceptibility of cells to redox imbalance-induced cell arrest.

Cell viability and cell cycle analysis

Table 1 summarizes the results on cell viability as measured by trypan blue dye exclusion 6 and 24 h after each treatment. There were no significant differences among the three treatment conditions, indicating that the inhibition of cell proliferation was not due to cell death. Moreover, cell viability as evaluated from the subG_0 fraction by flow cytometry ranged from 93.8% to 98.8% at 6 and 24 h for the three treatment condition groups (see **Fig. 6**), which corroborated the results of cell viability as measured by trypan blue exclusion. To determine the effect of redox imbalance on cell cycle changes, we subjected cells to flow cytometric analyses. Figure 6 illustrates the cell cycle profiles. At 6 h, there were no significant differences in the ratio of each phase of the cell cycle (i.e., G_0/G_1, S, and G_2/M) (Fig. 6). On the other hand, treatment with 30 μM diamide plus 30 μM BCNU resulted in a marked increase in the percentage of cells in the G_2/M phase at 24 h. Pretreatment of cells with BSO, followed by diamide plus BCNU, caused a further increase in the percentage of cells in the G_2/M phase (Fig. 6*A*, *B*). That the cell cycle did not change at 6 h, even though ^{3}H-thymidine incorporation was already decreased at this time, suggests that the effect of redox imbalance on cell proliferation was exerted at the G_1-to-S phase transition. The changes in cell cycle induced by GSH-to-GSSG shifts were partially reversed by 48 h in cells treated with diamide plus BCNU alone, but not in cells treated with the combination of BSO, followed by diamide plus

TABLE 1. *Effect of diamide, BCNU, and BSO on cell viability*[a]

Treatment	6 h	24 h
	(%)	(%)
Control	96.8 ± 0.9	96.0 ± 0.4
Diamide + BCNU	96.6 ± 1.5	95.7 ± 0.5
BSO + diamide + BCNU	94.7 ± 2.4	94.7 ± 0.6

[a] CaCo-2 cells were cultured in serum-containing DMEM and treated with 30 μM diamide plus 30 μM BCNU with or without pretreatment with 5 mM BSO. Incubations were for 6 or 24 h and cell viability was determined by trypan blue exclusion. BCNU was added 30 min before diamide treatment. BSO was administered 12 h before diamide treatment and was present throughout the incubation period. Cell viability was determined as the percentage of cells that excluded trypan blue. Results are the mean ± SE of 4 separate experiments.

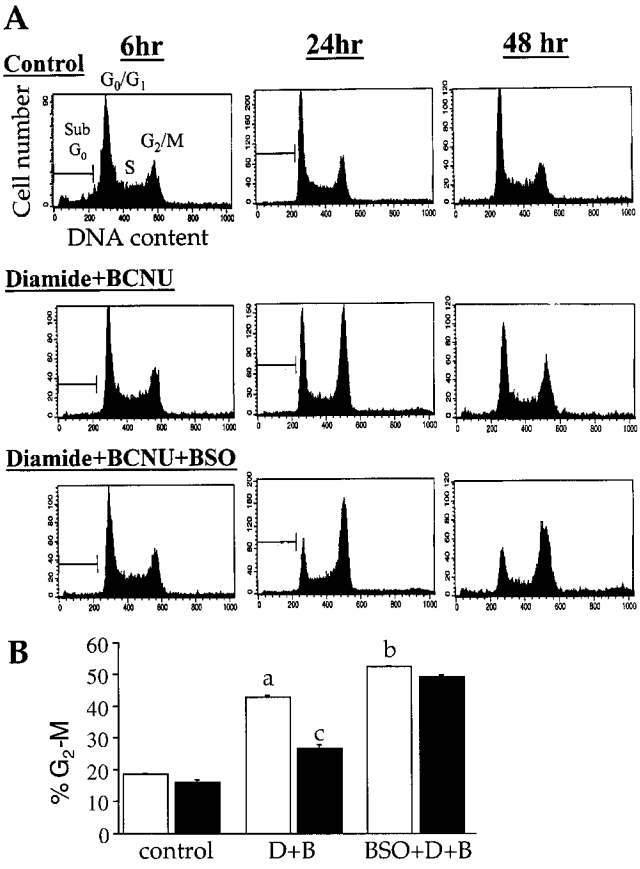

Figure 6. Cell cycle analysis by flow cytometry. Cells were cultured in serum-containing DMEM and treated with 30 μM diamide plus 30 μM BCNU with or without pretreatment of 5 mM BSO, and incubated for 6, 24, or 48 h. BCNU was added 30 min before diamide treatment. BSO was administered 12 h before diamide treatment and was present throughout the incubation period. *A)* Cell cycle analysis at 6 h, 24 h, and 48 h after treatment; *B)* percentages of cells in G_2/M phase to total cells at 24 h (open bar) and 48 h (filled bar). Panel *A* are representative results of 3 independent experiments; panel *B* are the mean ± SE of the results of 3 separate experiments. Control: no treatment, D+B: 30 μM diamide plus 30 μM BCNU, BSO+D+B: 30 μM diamide plus 30 μM BCNU after pretreatment of 5 mM BSO. a: $P < 0.05$ vs. 24 h control, b: $P < 0.05$ vs. 24 h control and D+B, c: $P < 0.05$ vs. 24 h D+B.

BCNU (Fig. 6*A*, *B*). Together, it appears that redox imbalance-mediated suppression of cell proliferation occurred in at least two phases of the cell cycle, namely, at the G_1-to-S phase transition and at the G_2/M phase. Moreover, the results show that a compromise in intracellular GSH level exaggerated and prolonged the cell cycle arrest.

ROS production by diamide, BCNU, and BSO

To verify that the effect of diamide plus BCNU was mediated by redox shift independent of oxyradical production secondary to decreased cellular GSH, we quantified oxidant production by measuring the oxidation of DHR. Oxidant formation was measured as the increase in fluorescence of rhodamine 123 after DHR oxidation (see Materials and Methods). **Table 2** summarizes the results of intracellular rhodamine 123 accumulation. There were no significant differences among the three treatment conditions at 6 and 24 h, indicating that neither treatment of cells with 30 μM diamide plus 30 μM BCNU nor with 5 mM BSO, followed by diamide plus BCNU, increased ROS production.

Effect of EGF on intracellular redox status and cell proliferation

EGF has been shown to exert mitogenic effect on serum-starved CaCo-2 cells (31), but not in serum-replete CaCo-2 cells (32). To test whether redox change is associated with the mitogenic effect of EGF, CaCo-2 cells were cultured in serum-free DMEM for 3 days before EGF treatment. Cells were incubated in serum-free DMEM with 100 ng/ml EGF alone or with EGF and 30 μM diamide plus 30 μM BCNU for 6 h. EGF treatment induced no significant changes in GSH or GSSG levels and did not change the GSH:GSSG ratio vs. control. Addition of 30 μM diamide plus 30 μM BCNU to EGF-treated cells shifted the GSH/GSSG thiol-disulfide balance in favor of GSSG at 30 min, although there was no statistical difference vs. EGF alone (**Fig. 7*A–C***). EGF increased ^{3}H-thymidine incorporation compared with control; this increase was abolished by the addition of diamide plus BCNU (Fig. 7*D*).

DISCUSSION

ROS and the accompanying oxidative stress are important stimuli that alter intracellular redox status. In our current study, we used a dose of diamide to change GSH-to-GSSG balance without the involvement of ROS (27, Table 2). At 30 μM, diamide did not change the intracellular GSH or GSSG level due to the rapid catalytic regeneration of GSH from GSSG by GSSG reductase. To achieve a more sustained redox shift, 30

TABLE 2. *Effect of diamide, BCNU, and BSO on DHR oxidation*[a]

Treatment	6 h	24 h
	(RFU/mg protein)	(RFU/mg protein)
Control	0.80 ± 0.037	1.61 ± 0.047
Diamide + BCNU	0.79 ± 0.040	1.68 ± 0.046
BSO + diamide + BCNU	0.88 ± 0.095	1.71 ± 0.034

[a] CaCo-2 cells were cultured in serum-containing DMEM and treated with 30 μM diamide plus 30 μM BCNU with or without pretreatment with 5 mM BSO. Incubations were for 6 or 24 h in the presence of 5 μM DHR. BCNU was added 30 min before diamide treatment. BSO was administered 12 h before diamide treatment and was present throughout the incubation period. Oxidant production was measured as the amount of rhodamine 123 formation. Results are the mean ± SE of 3 separate experiments.

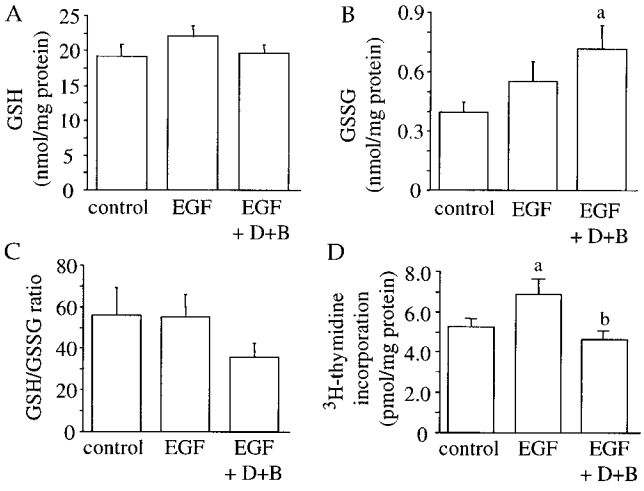

Figure 7. Effect of EGF on intracellular redox status and cell proliferation. Cells were cultured in serum-free DMEM for 3 days and treated with 100 ng/ml of EGF simultaneously with 30 μM diamide and incubated for 30 min in experiments A to C, or 6 h in experiment D. BCNU was added 30 min before diamide treatment. Control: no treatment, EGF: 100 ng/ml of EGF, EGF+D+B: 100 ng/ml of EGF+30 μM diamide plus 30 μM BCNU. *A)* GSH; *B)* GSSG; *C)* GSH:GSSG ratio; *D)* ^{3}H-thymidine incorporation. Results are the mean ± SE of 5 separate experiments. a: $P < 0.05$ vs. control. b: $P < 0.05$ vs. EGF.

μM BCNU was added to inhibit GSSG reductase and the conversion of GSSG to GSH (28). By the combined treatment of cells with 30 μM diamide and 30 μM BCNU, we were able to induce a marked and effective fall in the intracellular GSH:GSSG ratio (Fig. 1) with no detectable production of ROS (Table 2). The results from the current study have demonstrated a direct relationship between the induction of mild intracellular redox imbalance and the suppression of proliferation of the human colonic cancer CaCo-2 cells.

Previous studies have provided a link between intracellular GSH level and proliferating activity. Actively growing cells were associated with increases in total cellular GSH, and the decrease in GSH preceded the slowing of proliferating activity (15, 18). Other studies showed that maintenance of high GSH level was required for stimuli-induced cell proliferation (14, 19). However, the influences of GSSG and/or the ratio of oxidized-to-reduced glutathione on cell proliferation are poorly understood. In our study, treatment of cells with 30 μM diamide plus 30 μM BCNU decreased GSH and increased GSSG, resulting in a decreased GSH:GSSG ratio. Notably, this redox shift significantly inhibited cell proliferation as measured by changes in ^{3}H-thymidine incorporation, protein content, cell number, and cell cycle profiles (Figs. 2, 3, 6). The addition of NAC restored the intracellular redox balance and, correspondingly, cell proliferative activity (Fig. 4). Collectively, these results indicate that intracellular redox status is associated with cell proliferative activity that is independent of oxyradical production.

Evidence in the literature shows that a direct effect of depletion of glutathione level on cell proliferation is unresolved. Several studies have shown that BSO-induced glutathione depletion was sufficient to suppress cell proliferation (18, 33, 34), but others have reported that the loss of cell glutathione alone had no effect on cell proliferation (35–37). Our current study provided the distinction between the effect of GSH and the effect of GSH:GSSG ratio on the suppression of cell proliferation. BSO treatment alone markedly diminished intracellular GSH level but did not change the GSH:GSSG ratio at 30 min; this had a minimal effect on cell proliferation. On the other hand, treatment with diamide plus BCNU decreased GSH, increased GSSG, and lowered GSH:GSSG ratio within 30 min; this alteration in redox status resulted in inhibition of cell proliferation. Furthermore, our data show that the magnitude of suppression of cell proliferation after treatment with diamide plus BCNU was more severe when cells were first pretreated with BSO, although the GSH:GSSG ratios did not differ between the two treatment conditions (Fig. 5). These results mean that the initiation of inhibition of cell proliferative activity was mediated by an early and/or an abrupt decrease in GSH:GSSG ratio rather than by a depletion of GSH level. Moreover, whereas GSH depletion per se did not appear to have a direct suppressive effect on cell proliferation, a compromised GSH pool exacerbated the suppressive effects of redox imbalance on cell proliferation. This finding agrees with previous studies by Noelle et al. (38), who reported that diamide-induced inhibition of thymidine incorporation was more severe in cells with low GSH levels. White et al. (37) demonstrated that BSO by itself did not inhibit cell proliferation in bovine pulmonary artery endothelial cells, but BSO treatment enhanced the inhibition of cell proliferation induced by transforming growth factor β1.

A notable finding in this study is that a compromised intracellular GSH status renders cells more vulnerable to the suppressive effects of redox imbalance on cell proliferative activity. Moreover, redox-induced cell cycle arrest tended to be prolonged in cells with compromised cellular GSH, as evidenced by the delay in reversal of cell cycle changes in cells treated with a combination of BSO and diamide plus BCNU (Fig. 6). The reason low GSH exacerbates redox-induced suppression of proliferation is unclear. One possibility may be that a substantially decreased cellular GSH pool renders cells vulnerable to oxidizing events, as they are less able to maintain an intracellular reducing environment. Another interesting observation is that although the redox imbalance caused by diamide plus BCNU treatment occurred within 30 min, redox homeostasis returned to baseline level by 3 h, yet proliferation in these cells was more suppressed than confluent control cells at 24 h. This suggests that a sustained disruption of redox balance is neither necessary nor critical to effect the suppression of cell proliferation. Rather, our kinetic data studies are consistent with the conclusion that cell arrest was initiated by a rapid and abrupt fall in the GSH:GSSG ratio within minutes after oxidative challenge. Once initiated, the recovery of redox bal-

ance did not prevent the progression of cell arrest to its biological end point at 24 h. Furthermore, even though we found that BSO itself eventually caused a decrease in GSH:GSSG ratio at 24 h (there was no change in ratio at 30 min, Fig. 5*C*), this has little effect on cell proliferation. Collectively, the current results suggest that a transient disruption of redox homeostasis is critical to affect the proliferative outcome, and the temporal window between the loss of redox balance and the signal transduction that led to cell cycle arrest was within the first 30 min of exposure to redox agents. Recent studies from our laboratory also observed the effect of transient redox imbalance on apoptosis (6). In these latter studies, the magnitude of redox imbalance was significantly greater than that found to elicit cell arrest in the current study. These collective findings demonstrate that the phase transition of cells is differentially responsive to cellular redox status and that the magnitude of loss of redox balance may determine cell fate in favor of proliferation, differentiation, growth arrest, apoptosis, or necrosis.

The influence of redox imbalance in cell cycle progression is unclear. Recent studies have shown that diethylmaleate, which conjugates with and depletes GSH, activated the expression of $p21^{waf1}$ mRNA and produced G_1 and S phase delays and G_2/M arrest (39). It also induces a rapid dephosphorylation of the retinoblastoma protein, which is catalyzed by phosphatase independent of $p21^{waf1}$ accumulation (40). In other studies, hydrogen peroxide, which reacts with GSH and converts GSH to GSSG in a reaction catalyzed by GSH peroxidase, was found to cause G_2 phase arrest (41). In our study, the induction of redox shift by diamide plus BCNU did not cause a change in cell cycle progression at 6 h, although ^{3}H-thymidine incorporation was already suppressed at this time. This means that the G_1-to-S phase transition is a likely target site for redox (GSH/GSSG) control. At 24 h, redox shift clearly induced cell arrest at G_2/M. Together, these results show that redox imbalance likely exerts its effect at G_1-to-S phase transition and he G_2/M phase of the cell cycle. The mechanism by which changes in redox status inhibit cell proliferation remains to be identified. Several transcription factors, such as AP-1, NFκB, and p53, have been defined whose DNA binding activity and stimulatory effect on gene expression rely on the redox status of cysteinyl thiol in their structures (2). GSSG may interact in a reversible manner with protein cysteinyl thiol of many cellular proteins during oxidative stress (42). Indeed, GSSG may act as a protein phosphatase inhibitor via the formation of protein-disulfide cross-link in the phosphate transfer domain of the enzyme. Other studies have shown that GSH controls the onset of tumor cell proliferation by regulating protein kinase C activity and intracellular pH (34).

In our study, cells treated with diamide plus BCNU exhibited decreased ^{3}H-thymidine incorporation in both serum-containing medium and serum-free medium. In contrast, ODC activity was suppressed by diamide plus BCNU treatment only in serum-free medium. The reason for this interesting observation is unclear. Typically, ODC is expressed in growing CaCo-2 cells and plays a major role in cell replication (43), and ODC activity has been shown to be modulated by medium condition in vitro (43) or food in vivo (23). This difference in ODC activity in response to redox challenge may be due to stimulatory effects of trophic factors or different nutrients under serum-replete but not serum-depleted conditions.

Literature evidence shows that some growth factors affect intracellular redox status. For example, keratinocyte growth factor was found to modulate cellular GSH/GSSG redox status in the intestinal mucosa of food deprived-refed rats (44), and platelet-derived growth factor can increase cellular GSH content (21). Smith et al. reported that growth factors that promote self-renewal, such as neurotrophin-3 and basic fibroblast growth factor, induce a greater reduced intracellular redox status. Pharmacological antagonists of these reducing effects were found to inhibit self-renewal (8). In our current study, EGF stimulated ^{3}H-thymidine incorporation, and this increase was abolished by induction of redox imbalance with diamide plus BCNU. The proliferative effect of EGF was not associated with intracellular redox change, in agreement with previous studies (21).

In summary, we have shown that intracellular redox imbalance inhibits cell proliferation independent of ROS production. Depletion of GSH per se has no effect on cell proliferation, but a compromised baseline cellular GSH exacerbated the susceptibility of cells to redox imbalance-induced inhibition of proliferation. Our data support the conclusion that an early and transient fall in GSH:GSSG ratio initiated the suppression of proliferative activity. Notably, cell cycle analyses revealed that the G_1-to-S phase transition and the G_2/M phase are potential targets for redox regulation. Although EGF stimulated cell proliferation, this effect was unrelated to changes in the intracellular thiol-disulfide status. However, EGF-induced cell proliferation was readily abolished by the induction of cellular redox imbalance. FJ

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