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Nitrite-Mediated Modulation of HL-60 Cell Cycle and Proliferation: Involvement of Cyclin-Dependent Kinase 2 Activation

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ABSTRACT

Recent research suggests the vivid possibility of using nitrite therapy against various pathological conditions. Moreover, chronic nitrite therapy offers protection against ischemia and augments endothelial cell proliferation through unknown mechanisms. Nitrite-mediated augmentation in the number of circulating neutrophils has also been reported; however, the exact mechanism is not known. In the present study, we have investigated the effect of nitrite (0.5–10 mM) on the proliferation of the neutrophilic cell line HL-60 and also explored the underlying mechanism. Treatment of HL-60 cells with sodium nitrite (0.5–5 mM) led to an increase in cell proliferation, which was confirmed by cell cycle analysis and 5-bromo-2-deoxyuridine and thymidine incorporation, whereas cells accumulated in the G_0/G_1 phase after treatment with 10 mM nitrite. Experiments on the synchronized cells exhibited similar effect, which seems to be nitric oxide (NO)-dependent, because carboxyl-1*H*-imidazol-1-yloxy,2-(4-carboxyphenyl)-4,5-dihydro 4,4,5,5-tetramethyl-3-oxide abolished nitrite-mediated proliferative effect. Moreover, the NO donor sodium nitroprusside at micromolar concentrations also exhibited similar effects. Nitrite induced augmentation in S phase, and intracellular reactive oxygen species (ROS) generation was prevented by ROS scavenger/inhibitors. Moreover, mitochondrial blockers, rotenone and antimycin A, also reduced nitrite-mediated cell proliferation. Assessment of the cell cycle regulators cyclin-dependent kinase 2 (Cdk2), Cdk4, cyclin A, cyclin D, cyclin E, and p21 suggested augmentation in the expression and interaction of Cdk2/cyclin E and Cdk2 activity, whereas p21 was down-regulated. Indeed proliferative effect of nitrite was blocked by roscovitine, a Cdk2 inhibitor. The results obtained demonstrate that the proliferative effect of nitrite on HL-60 cells seems to be NO-mediated, redox-sensitive, and Cdk2 activation-dependent, warranting detailed studies before initiating its clinical use.

Introduction

The vasodilatory properties of nitrite have been known for more than half of a century (Lundberg and Weitzberg, 2009); however, after discovery of mammalian nitric-oxide (NO) synthase enzymes in the late 1980s, nitrite was mostly considered biologically as an inactive end product of NO metabolism (Knowles and Moncada, 1994). The most common use of nitrate salts is as an antidote against cyanide poisoning and for curing foods to impart a pleasant color to meat and prevent botulism (Gladwin et al., 2005; Butler and Feelisch, 2008). Under certain conditions, nitrites mediate formation of nitrosamines, which are potent carcinogens (Tannenbaum et al., 1976). Studies have found no direct correlation of nitrite-mediated formation of *N*-nitrosamines with carcinogenesis (Lundberg and Weitzberg, 2009). Moreover, research has also suggested a role for nitrite in important physiological and pathophysiological functions (Gladwin et al., 2005; Butler and Feelisch, 2008; Lundberg et al., 2008).

NO is intricately involved in assisting physiological processes such as host defense, vascular tone, proliferation, and apoptosis (Bogdan, 2001). Hemoglobin, myoglobin, or other metal-containing enzymes, under hypoxic or ischemic conditions catalyze the reduction of nitrite to NO (Cosby et al., 2003; Gladwin et al., 2005; Dezfulian et al., 2007). Nitrite has even been proposed to be a signaling molecule under physiological as well as ischemic conditions (Bryan et al., 2005).

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ABBREVIATIONS: NO, nitric oxide; BrdU, 5-bromo-2-deoxyuridine; Cdk, cyclin-dependent kinase; DTT, dithiothreitol; NAC, *N*-acetyl-L-cysteine; ROS, reactive oxygen species; cPTIO, carboxyl-1*H*-imidazol-1-yloxy,2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5- tetramethyl-3-oxide; PI, propidium iodide; SNP, sodium nitroprusside; DAF, 4,5-diamino-fluorescein; 7-AAD, 7-aminoactinomycin D; DPI, diphenyleneiodonium; FITC, fluorescein isothiocyanate; RB, retinoblastoma; FACS, fluorescence-activated cell sorting; DCF, dichlorofluorescein; DHE, dihydroethidium.

Nitrite also serves a diagnostic marker and plays a role as a potential therapeutic agent in offering protection against myocardial and liver ischemia/reperfusion-induced injury (Webb et al., 2004; Bryan et al., 2007). Moreover, the use of chronic dietary nitrite supplementation (50 mg/liter) in drinking water for 1 week restored NO homeostasis in eNOS(−/−) mice and protected them against ischemia and reperfusion injury (Bryan et al., 2008). Nitrite therapy significantly augmented vascular density in the ischemic limbs by inducing endothelial cell proliferation and angiogenesis (Kumar et al., 2008); however, underlying mechanisms were not investigated. Nitrite also prevented endothelial cell apoptosis after UV exposure (Suschek et al., 2003). Oral administration of sodium nitrite (25–100 mg/kg) reduced lymphocytes, but augmented circulating neutrophils in BALB/c mice (Abuharfeil et al., 2001). Later, reduction in lymphocytes was confirmed by Ustyugova et al. (2002). Recent research suggests a vivid possibility that nitrite therapy might be effective against various pathological conditions, but this seems to be a precipitate verdict.

Cell cycle and proliferation are under redox regulation (Felty et al., 2005; Lu et al., 2007; Menon and Goswami, 2007; Kumar et al., 2010). It is noteworthy that growth factors stimulate rapid increase in intracellular ROS that modulate signaling involved in cell growth (Sattler et al., 1999; Felty et al., 2005). The G_1- to S-phase transition in fibroblasts depends on the intracellular redox status (Menon et al., 2003). Mitochondria have been found to play a central role in cell cycle regulation (Mitra et al., 2009). Moreover, disruption of the mitochondrial electron transport chain specifically retards the G_1- to S-phase transition (Felty et al., 2005; Owusu-Ansah et al., 2008). Incidentally, we and others have reported that mitochondria are potential NO targets and affect ATP generation, apoptosis, and cell cycle (Brookes et al., 2000; Kumar et al., 2010).

We have demonstrated a biphasic regulation of the HL-60 cell cycle and proliferation by NO that was mediated by cyclin-dependent kinase 2 (Cdk2) nitrosylation, whereas apoptosis was caused by the loss of mitochondrial potential (Kumar et al., 2010). Augmentation of neutrophil number has been reported after nitrite treatment (Abuharfeil et al., 2001). The present study was therefore undertaken to investigate the effect of sodium nitrite on cell cycle and cell proliferation of HL-60, a promyelocytic cell line, which has been extensively used to assess neutrophil proliferation, differentiation, and functions (Kanayasu-Toyoda et al., 1999; Drayson et al., 2001; Schaff et al., 2010). Moreover, we also explored the mechanisms involved in nitrite-mediated proliferation. We observed that nitrite treatment (0.5–5 mM) augmented HL-60 cell proliferation and S phase of the cell cycle in a concentration-dependent manner, whereas treatment with 10 mM nitrite led to cytostasis. The proliferative effect of nitrite further seems to be NO-mediated and also dependent on ROS and mitochondria. Modulation of the cell cycle regulators, especially Cdk2/cyclin E interaction and augmented Cdk2 activity, was observed; indeed, roscovitine pretreatment inhibited the proliferative effect of nitrite. The present study explores nitrite-mediated cell cycle regulation of HL-60 cells and proposes the involved putative mechanisms.

Materials and Methods

Chemicals and Reagents. Sodium nitrite, sodium nitrate, 7-aminoactinomycin D (7-AAD), *N*-acetyl-L-cysteine (NAC; ROS scavenger), antimycin A (complex III inhibitor), carboxyl-1*H*-imidazol-1-yloxy,2-(4-carboxyphenyl)-4,5-dihydro 4,4,5,5-tetramethyl-3-oxide (cPTIO; NO scavenger), dithiothreitol (DTT), diphenyleneiodonium (DPI), L-mimosine, propidium iodide (PI), and rotenone (complex I inhibitor) were purchased from Sigma-Aldrich (St. Louis, MO). 6-(Benzylamino)-2(*R*)-[1-(hydroxymethyl) propyl amino]-9-isopropyl purine (roscovitine; Cdk2 inhibitor) was procured from Calbiochem (San Diego, CA). The annexin V FITC kit and 5-bromo-2-deoxyuridine (BrdU) FITC kit were purchased from BD Biosciences (San Diego, CA).

Cell Culture and Nitrite Treatment. HL-60 cells (American Type Culture Collection, Manassas, VA) were grown in RPMI medium 1640 containing $NaHCO_3$, 2 mM glutamine, 10% (v/v) fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in a 5% CO_2 atmosphere (Kumar et al., 2010). Cells were seeded at a concentration of 2×10^5 cells/ml and maintained for logarithmic growth by passaging them every 2 to 3 days and incubating them for 1 to 3 days with sodium nitrite at various concentrations (0.5–10 mM). To explore the putative mechanism, cells were pretreated with various interventions/vehicles such as cPTIO (a NO scavenger), DTT, ROS inhibitors, mitochondrial blockers (rotenone and antimycin A), and Cdk2 inhibitor (roscovitine) before nitrite treatment as mentioned in the figure legends.

Cell Viability Assessment. Vehicle or nitrite-treated HL-60 cells (1×10^5cells/ml) in the culture medium were directly incubated with PI (5 μg/ml in phosphate-buffered saline) after the completion of incubation periods to avoid any adverse effect on viability during centrifugation/washing. Samples were examined by acquiring 20,000 cells after 10 min of staining and subsequently analyzed using the Cell Quest 5.2 program (FACS Calibur; BD Biosciences). Annexin V labeling of cells was also performed using an annexin V FITC kit (BD Biosciences) (Kumar et al., 2010).

Cell Cycle Analysis. DNA staining (Krishan, 1975) was performed in cells treated with control/vehicle, nitrite, sodium nitroprusside (SNP), or the various interventions mentioned in the figure legends. In brief, HL-60 cells (1×10^5) were centrifuged at 150*g* for 5 min and the pelleted cells were resuspended in hypotonic propidium iodide solution (50 μg/ml with 0.03% NP-40 in 0.1% sodium citrate). Samples were acquired after 10 to 20 min of staining at 4°C. DNA content in these cells was assessed using a flow cytometer (FACS Calibur; BD Biosciences) and the Cell Quest program, and cell-phase distribution was analyzed by Modfit 3.0 software (Verity Software House, Topsham, ME).

Cell Synchronization. L-Mimosine was used for cell synchronization at the G_0 phase as described previously (Krude, 1999). Synchronization of HL-60 cells was achieved by adding mimosine, which was assessed by flow cytometry using isolated nuclei stained with propidium iodide (50 μg/ml with 0.03% NP-40 in 0.1% sodium citrate) and analyzed by FACS Calibur (BD Biosciences). To explore the effect of sodium nitrite on cell cycle progression in the synchronized cells, mimosine was removed by washing the cells with RPMI medium and cells were incubated with various concentration of sodium nitrite.

[^{3}H]Thymidine Incorporation Assay. HL-60 cells (1×10^5 cells) were cultured in 96-well plates in RPMI medium 1640 with 10% fetal bovine serum, and sodium nitrite was added at 1 mM concentration. [^{3}H]Thymidine (1 μCi) was added to the plates in the last 24 h and cultured in 5% CO_2 at 37°C. After 24, 48, and 72 h, the cells were harvested on a semiautomated cell harvester (Biotron Healthcare, Mumbai, India), and the radioactivity was measured with a scintillation counter (PerkinElmer Life and Analytical Sciences, Waltham, MA). All experiments were performed in triplicate.

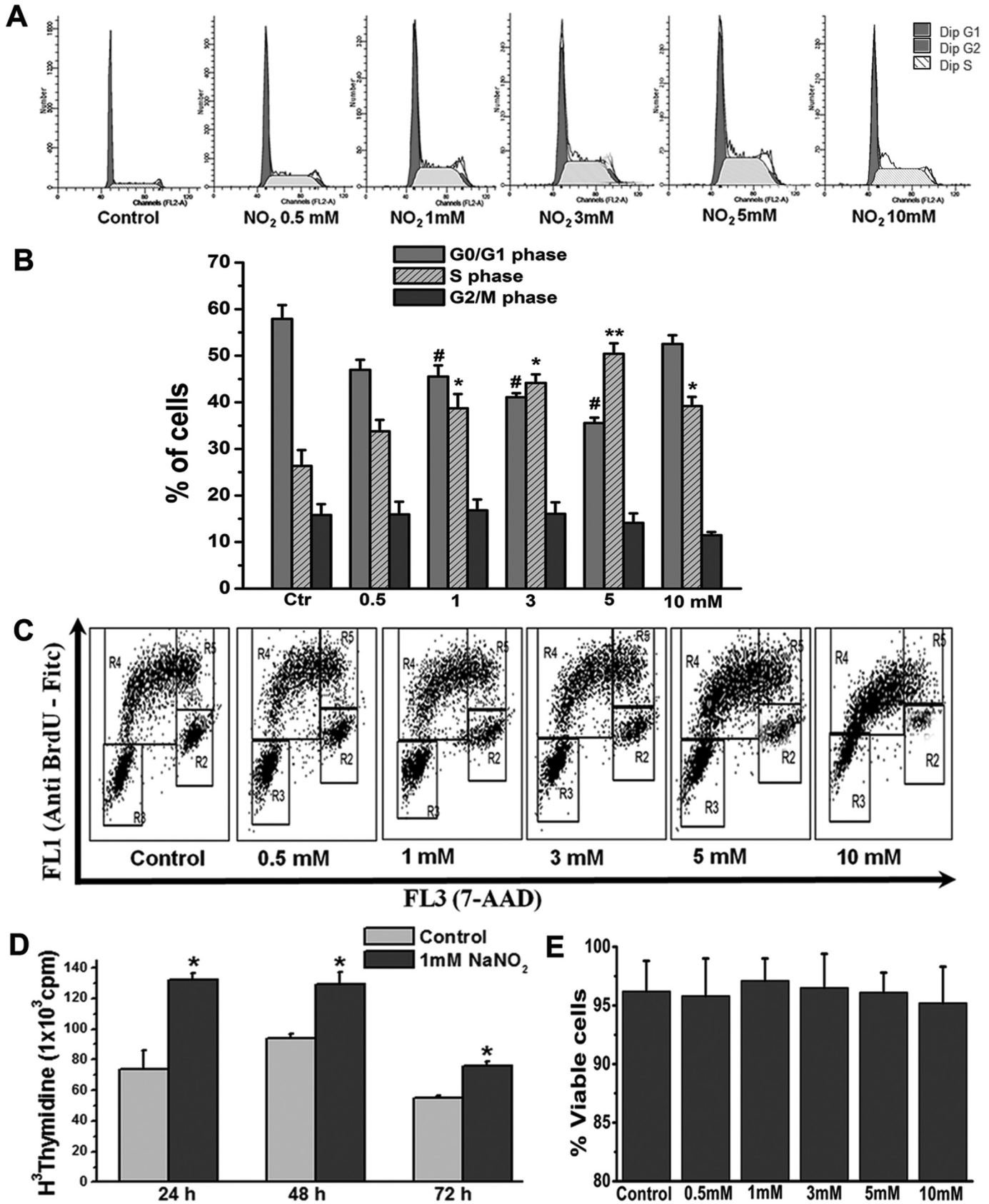

Fig. 1. Effect of sodium nitrite on cell cycle and proliferation of promyelocytic cell line. A, DNA content of control and nitrite-treated cells after 24-h treatment using hypotonic propidium iodide solution (50 μg/ml with 0.03% NP-40 in 0.1% sodium citrate). Cell cycle-phase distribution was analyzed by Modfit software (Verity Software House). B, quantitative data of cell cycle distribution in control and nitrite-treated cells. *, $p < 0.01$; * *, $p < 0.001$ in comparison with S phase of control. #, $p < 0.01$ in comparison of G_1 phase with control. C, dot plots exhibiting BrdU incorporation in control and nitrite-treated cells (0.5–10 mM) after 24 h. The *y*-axes show significantly more cells in S phase (DNA synthesis) after nitrite treatment. D, HL-60 cells

BrdU Incorporation Assay. DNA synthesis during S phase was assessed by measuring the incorporation of BrdU into DNA using a cell proliferation kit (BrdU FITC Kit; BD Biosciences) according to the manufacturer's instructions. In brief, cells were pulsed with 10 μM BrdU for 30 min in culture followed by fixation with BD Cytofix/Cytoperm buffer, permeabilization by BD Cytoperm plus buffer, DNAase treatment, and detection with anti-BrdU- FITC antibody. DNA was simultaneously stained with 7-AAD. Cells were analyzed on a FACS Calibur.

Estimation of NO Release from Nitrite. 4,5-Diamino-fluorescein (DAF) fluorescence time kinetics was performed to explore the NO release from nitrite in the present experimental condition. DAF reacts rapidly and irreversibly with NO to produce a highly reactive fluorescent product triazolo fluorescein (DAF-2T). The NO donor SNP was used as standard. DAF (10 μM) was incubated with the reaction system for 10 min at 37°C to stabilize the basal fluorescence. DAF fluorescence was measured for 150 min after nitrite or SNP treatment at 37°C using wavelength 480 nm (excitation) and 523 nm (emission) by fluorimeter (Varian Caryeclipse; Varian, Inc., Palo Alto, CA).

Western Blot Analysis. Cells were lysed with protein extraction buffer/lysis buffer (20 mM Tris, pH 7.5 containing 1% Triton X-100, 150 mM NaCl, 1 mM Na_3VO_4, 1 mM NaF, and protease inhibitors). Protein concentrations were measured with a BCA protein assay reagent kit (Thermo Fisher Scientific, Waltham, MA). Samples containing equal amounts of protein (40 μg) were run on 10 to 12% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Blots were probed with antibodies specific for cyclin A, cyclin E, cyclin D, p21, Cdk2, and Cdk4 (Cell Signaling Technology, Danvers, MA) or actin (Sigma-Aldrich) at 1:1000 dilution in 25 mM Tris, 150 mM NaCl, and 0.1%Tween 20. Furthermore, appropriate horseradish peroxidase-conjugated secondary antibodies (Sigma-Aldrich) were used. Chemiluminescent visualization of proteins was done by using ECL-plus (GE Healthcare) (Kumar et al., 2010).

Cdk2 Kinase Assay. In vitro Cdk2 kinase activity was measured as described previously (Kumar et al., 2010). The cell extracts were obtained by using cell lysis buffer (50 mM Tris-HCl, pH 7.0, 150 mM NaCl, 5 mM EDTA, pH 8.0, 0.5% NP-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaF, 20 mM sodium pyrophosphate, 1 mM sodium vanadate, and 10 μg/ml each of leupeptin, aprotinin, and pepstatin) on ice for 30 min. Total 200 μg of protein lysate, after preclearing with equilibrated protein A beads, were incubated overnight with 1 μg of mouse anti-Cdk2 IgG (BD Biosciences) at 4°C followed by immunoprecipitation with protein A agarose conjugate at 4°C for 2 h. The beads were washed three times with kinase buffer (50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, and 1 mM DTT). The Cdk2 kinase reaction were performed at 30°C for 30 min in kinase buffer containing 5 μg of retinoblastoma (RB) protein (amino acids 773–928; Millipore Corporation, Billerica, MA) as substrate and 100 μM ATP. The reaction was stopped by adding 2× Laemmli buffer and boiled for 5 min. Phosphorylation of RB was analyzed on 10% SDS-polyacrylamide gel electrophoresis followed by Western blotting with p(Ser) Cdk substrate antibody (Cell Signaling Technology), which detected phosphorylation of RB protein.

Assessment of Intracellular GSH. Cells were stained with monobromobimane (40 μM) for 10 min at room temperature and run on a FACS Aria (BD Biosciences) with excitation at 353 to 361 nm and emission at 450 nm (Kumar et al., 2010). An increase in fluorescence correlated with GSH content. A replicate sample was depleted of GSH by treatment with 100 μM *N*-ethylmaleimide to assess nonspecific binding of the monobromobimane probe. GSH content was assessed in a minimum of 10,000 cells.

Statistical Analysis. Data are presented as mean ± S.E.M., of at least three to five independent experiments and were analyzed by one-way analysis of variance test followed by Newman-Keuls post-analysis. Student's *t* test analysis was also used to compare control versus treated cells as specified in the figure legends. Data were considered significant at $p < 0.05$.

Results

Nitrite-Modulated Cell Cycle and Proliferation. Treatment of promyelocytic HL-60 cells with sodium nitrite (0.5–10 mM) for 24 h augmented the S phase of the cell cycle significantly (Fig. 1, A and B). Similar effects were observed at 48 and 72 h (data not shown). The percentage of cells in each phase, as calculated by using Modfit software, and its relationship to the concentration of nitrite is illustrated in Fig. 1B. To ensure the specificity of nitrite, sodium nitrate was used; it had no effect on the HL-60 cell cycle (data not shown). S-phase augmentation was further confirmed by BrdU incorporation as new DNA synthesis in nitrite-treated cells (Fig. 1C). Moreover, HL-60 cells cultured in the presence of 1 mM nitrite for 24 to 72 h exhibited proliferation, as demonstrated by an increase in [^{3}H]thymidine incorporation (Fig. 1D). Under the experimental conditions used there was no loss in cell viability (PI-permeable) at all of the concentrations of nitrite (Fig. 1E). Moreover, annexin V labeling revealed only 4 to 6% annexin V-positive cells in control- and nitrite-treated cells. However, treatment of cells with 10 mM sodium nitrite showed a trend of cell accumulation in the G_0/G_1 phase, even though there were significantly more cells in the S phase in comparison with the vehicle-treated control cells (Fig. 1A). To establish the proliferative effect of sodium nitrite on HL-60 cells further experiments were performed at 1 to 5 mM concentration.

Effect of Nitrite on Synchronized Cells. Synchronized cells were also used to validate nitrite-mediated modulation of the cell cycle. HL-60 cells were synchronized in the G_1 phase with mimosine (0.5 mM) for 18 h to confirm the proliferative effect (Fig. 2A). Mimosine-mediated block was reversible because removal of mimosine led to the re-entry of cells into S and G_2M phases (Fig. 2B), but a substantial number of cells died. The addition of nitrite to G_1-synchronized cells demonstrated entry of more cells in S phase after 24-h treatment using nitrite (Fig. 2C), which was also confirmed by BrdU incorporation (Fig. 2D).

The Role of NO in Mediating Effect of Nitrite. Nitrite-mediated effects under both physiological and pathophysiological conditions have been shown to be caused primarily by the reduction of nitrite to NO (Webb et al., 2004; Bryan et al., 2008; Butler and Feelisch, 2008). We therefore explored the involvement of NO in nitrite-mediated cell cycle modulation. The increase in S phase by nitrite treatment was abolished in the presence of 300 μM NO scavenger cPTIO, suggesting that NO could be a mediator of nitrite effect (Table 1). Addition of

were cultured in 96-well plates with nitrite (1 mM). Thymidine incorporation was accessed after harvesting the cells at different time intervals on a Biotron semiautomatic cell harvester; radioactivity was quantified with a scintillation counter. [^{3}H]Thymidine (1 μCi) was added to the cells in the last 24 h of nitrite treatment in 5% CO_2 at 37°C. Data are presented as means ± S.E.M. of at least five experiments. *, $p < 0.01$ in comparison with the control. E, cell viability was analyzed by quantifying the PI-impermeable cells in control and nitrite-treated cells by using flow cytometry as described under *Materials and Methods*.

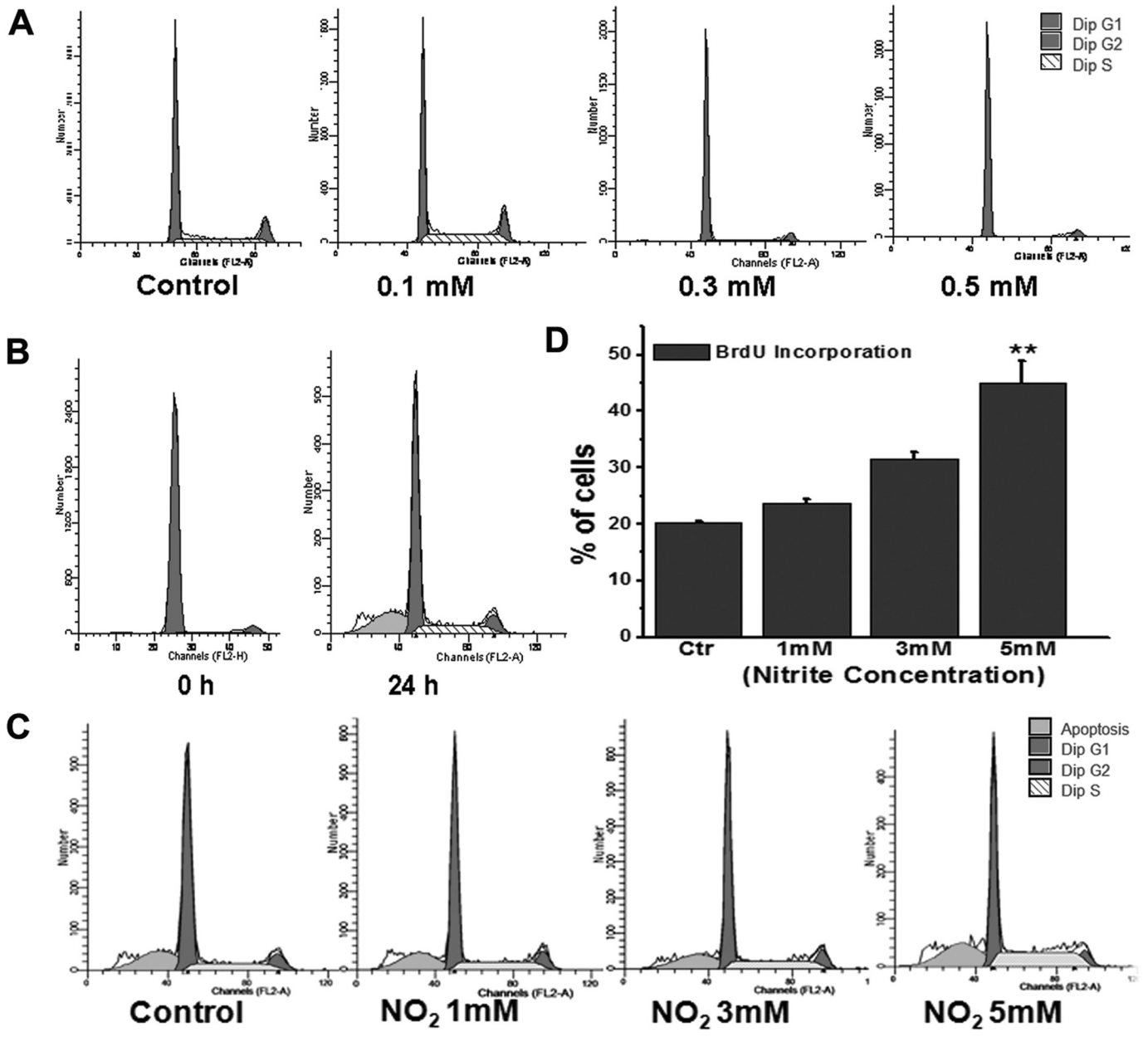

Fig. 2. Effect of sodium nitrite on synchronized HL-60 cells. A, cells were synchronized in G_1 phase with mimosine (0.1–0.5 mM) for 18 to 24 h. B, this block was reversible, because cells cultured for 24 h without mimosine reverted to proliferation. C, synchronized cells treated with nitrite (1–5 mM) in fresh medium for 24 h were acquired after PI staining and subsequently analyzed with Modfit software. D, nitrite-treated synchronized cells were pulsed with BrdU for 30 min as described under *Materials and Methods*. **, $p < 0.001$ in comparison with controls versus nitrite treatment.

TABLE 1

Effect of c-PTIO on nitrite-mediated cell cycle modulation

Cells were treated with 300 μM c-PTIO for 30 min before nitrite addition (1–5 mM). Distribution of cells in various phases of the cell cycle was analyzed by Modfit software. Data are presented as percentage of cells. Means ± S.E.M. of five individual experiments.

	Control			cPTIO		
	G_0/G_1	S	G_2/M	G_0/G_1	S	G_2/M
			%			
Control	58 ± 3	29 ± 2	13 ± 2	62 ± 4	26 ± 2	12 ± 1
0.5 mM Nitrite	54 ± 3	32 ± 3	14 ± 2	62 ± 3	26 ± 3	12 ± 2
1 mM Nitrite	49 ± 4	36 ± 3*	15 ± 3	62 ± 3	27 ± 3@	11 ± 3
3 mM Nitrite	42 ± 3	43 ± 5**	15 ± 2	61 ± 2	28 ± 2@	11 ± 2
5 mM Nitrite	38 ± 4	48 ± 5**	14 ± 3	59 ± 4	29 ± 3@	12 ± 3

* $P < 0.01$; ** $P < 0.001$ in comparison of S-phase cells in control vs. after nitrite treatment. @ $P < 0.01$ in comparison of nitrite vs. c-PTIO treatment.

the slow NO releaser SNP also exhibited a proliferative effect on HL-60 cells at 10 to 100 μM concentration (Fig. 3 A). To investigate nitrite-mediated NO release, we monitored kinetics of DAF fluorescence to assess NO release from nitrite and SNP. In the present experimental condition, 5 mM nitrite yielded a DAF-2T signal similar to that obtained with 50 μM SNP to implicate the release of NO (Fig. 3B). The effect of nitrite on the cell cycle seems to be caused by NO, which was also confirmed by pretreatment with DTT (1 mM), which inhibited NO-mediated events (Fig. 3D). Furthermore GSH content was also found to be significantly augmented in nitrite-treated cells (Fig. 3C), suggesting the redox regulation in nitrite-mediated proliferation.

The Role of ROS and Mitochondria in Modulating the Nitrite-Mediated Cell Cycle. ROS regulate cellular

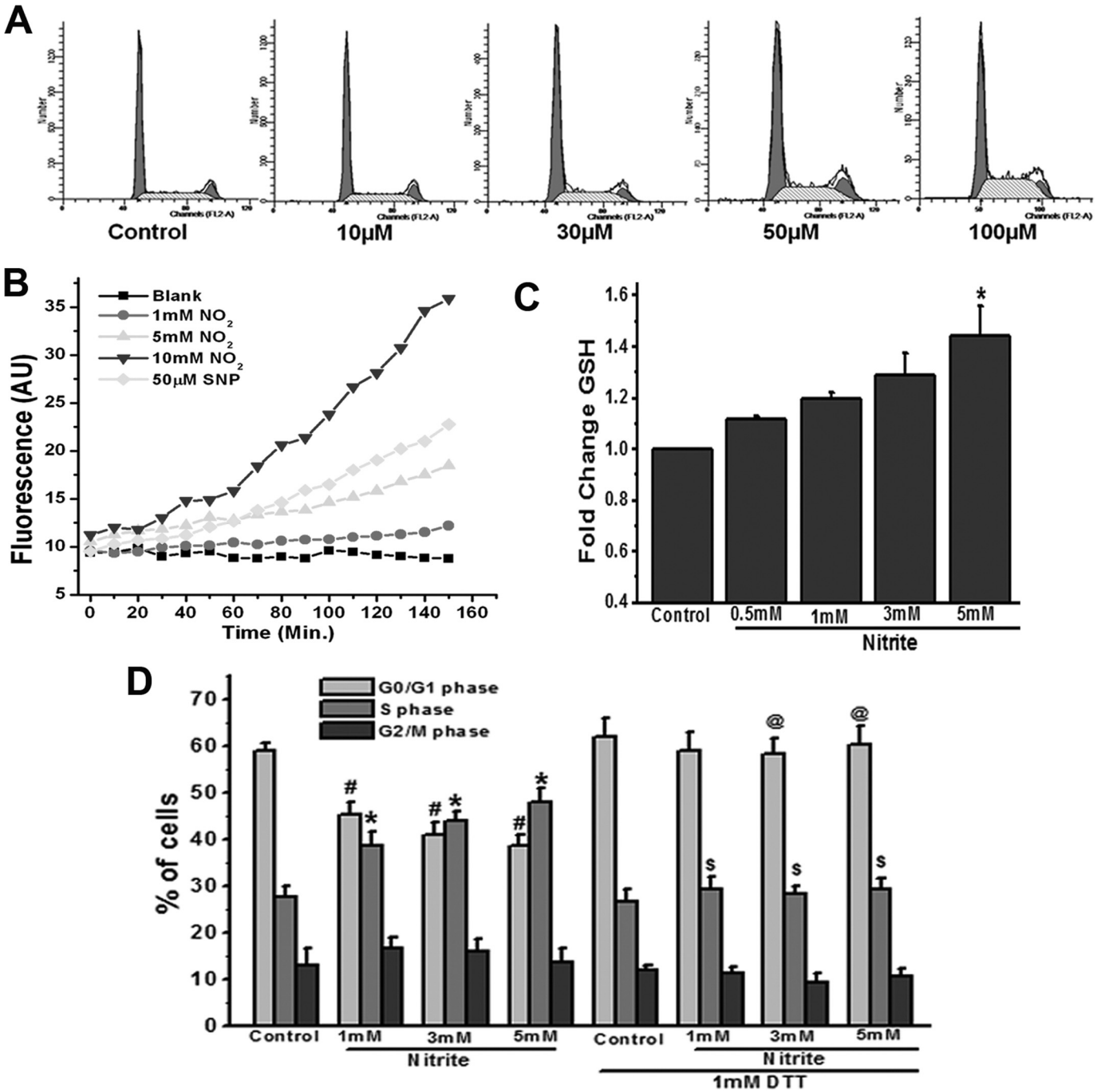

Fig. 3. Effect of SNP on HL-60 cells, nitrite-mediated effect on GSH, and modulatory effect of DTT. A, cell cycle-phase distribution after 10 to 100 μM SNP treatment of HL-60 cells for 24 h. B, nitrite-mediated NO release in present experimental conditions was assessed by DAF fluorescence kinetics. The NO donor SNP was used as reference NO releaser. C, GSH content after treatment of HL-60 cells at different concentrations of nitrite (1–5 mM). Data are presented for three individual experiments as fold change in fluorescence of nitrite-treated cells versus controls. *, $p < 0.01$ in comparison with control cells versus nitrite treatment. D, treatment of cells with 1 mM DTT for 30 min before nitrite addition (1–5 mM) and cell cycle was analyzed. Data are presented as percentage of cells. Means ± S.E.M. of five individual experiments. #, $p < 0.01$ in comparison of the G_0/G_1-phase cells in controls versus nitrite-treated cells. *, $p < 0.001$ in comparison of the S-phase cells in controls versus nitrite treatment. @, $, $p < 0.01$ in comparison of the G_0/G_1 and S-phase cells, respectively, in DTT versus nitrite treatment.

signaling and cell proliferation (Sattler et al., 1999; Felty et al., 2005). We therefore investigated the possibility that nitrite might induce ROS generation in HL-60 cells and modulate cell cycle/proliferation. ROS level was enhanced in nitrite (0.5–5 mM)-treated cells in a concentration-dependent manner as assessed by DCF (a broad range ROS detector) and DHE (a superoxide-specific dye) (Fig. 4, A and B). Furthermore, free radical scavenger NAC or inhibitor DPI pretreatment significantly reduced nitrite-mediated augmentation in the S phase and cell proliferation to the vehicle-treated control level (Fig. 4C). Mitochondria have been identified to play a key role during S-phase transition and are also an important target of NO action. We thus explored the effect of mitochondrial function blockers on nitrite-mediated cell cycle modulation. HL-60 cells pretreated for 1 h with specific blockers of respiratory complex I (rotenone) and complex III (antimycin A) exhibited prevention of S-phase induction by nitrite (Fig. 4C). There were more cells in S phase in antimycin A treatment, but nitrite-mediated increase in S phase was not observed. Moreover, free radical modulators and mitochondrial inhibitors significantly reduced nitrite-mediated augmentation in ROS to the vehicle-treated control level (data not shown). Together, these findings suggest that nitrite-mediated cell cycle S phase of HL-60 cells also depended on mitochondria-mediated ROS generation.

Effect of Nitrite on Cyclin/Cdk Expression and Involvement of Cdk2. Expression of various cyclins/Cdks, which regulate the progression of cells from G_1 to S phase, such as cyclin A, cyclin D, cyclin E, Cdk2, Cdk4, and p21 were monitored (Fig. 5, A and B). Western blotting of these proteins after treatment of sodium nitrite exhibited significant enhancement of cyclin A, cyclin D, cyclin E, Cdk2, and Cdk4 expression at 1 to 5 mM nitrite, whereas expression of cell cycle inhibitor p21 was reduced. These results overall represent more active cell cycle machinery in the proliferating cells after treatment with 1 to 5 mM nitrite. However, treatment with 10 mM nitrite significantly down-regulated the expression of these cell cycle regulators. It has been reported that appropriate temporal activation and interaction of cyclin E/Cdk2 is required for progression through the G_1 and S entry, whereas p21/Cdk2 interaction inhibits cell cycle progression. Moreover, Cdk2 and cyclin E interaction was also investigated and found to be augmented in up to 5 mM sodium nitrite-treated cells (Fig. 5, C and D). Furthermore, Cdk2 kinase activity was measured by using RB protein, a substrate of Cdk2, which was augmented at 1 to 5 mM nitrite concentration (Fig. 5E). Increase in Cdk2 activity did not depend on enhanced Cdk2 expression as evident by the activity/expression ratio (Fig. 5F). However, sodium nitrite (10 mM) rescued cyclin E/Cdk2 interaction and Cdk2 kinase activity as well, suggesting cytostasis; no cell death was observed.

Furthermore, the most commonly used purine analog, roscovitine, a potent and selective inhibitor of cyclin-dependent kinases with high specificity toward Cdk2, was used to explore the role of Cdk2 in nitrite-mediated cell cycle regulation. Of the doses of 10, 20, and 50 μM, 20 μM roscovitine was used in the present study, 20 μM was the optimal concentration, and 50 μM induced cell death (data not shown). Roscovitine (20 μM) prevented nitrite (1–5 mM)-mediated S-phase

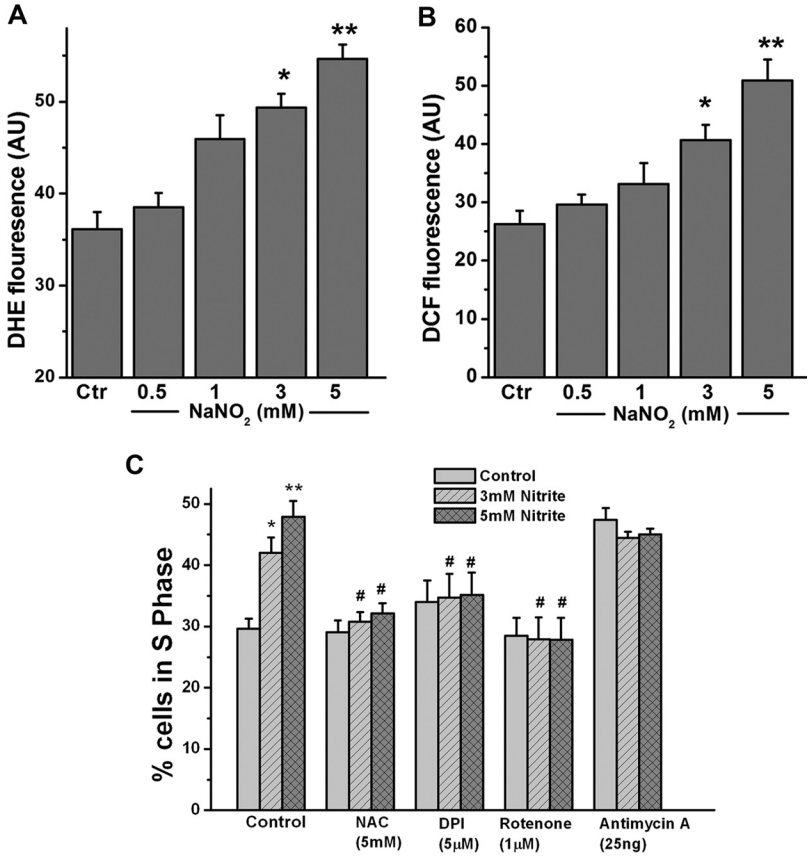

Fig. 4. Involvement of ROS and mitochondria in nitrite-mediated effect. A and B, 1×10^6 cells were incubated with 10 μM DCF-DA (A) or 10 μM DHE (B) for 30 min at 37°C, and 10,000 cells from each tube were acquired and analyzed by a flow cytometer using the Cell Quest program. *, $p < 0.01$; **, $p < 0.001$ in comparison with vehicle versus nitrite treatment. C, cell-cycle S phase was analyzed by Modfit software (Verity Software House) in cells preincubated with NAC, DPI, rotenone, or antimycin A before nitrite treatment. *, $p < 0.01$; * *, $p < 0.001$ in comparison with controls and S phase. #, $p < 0.01$ in comparison with nitrite-treated cells.

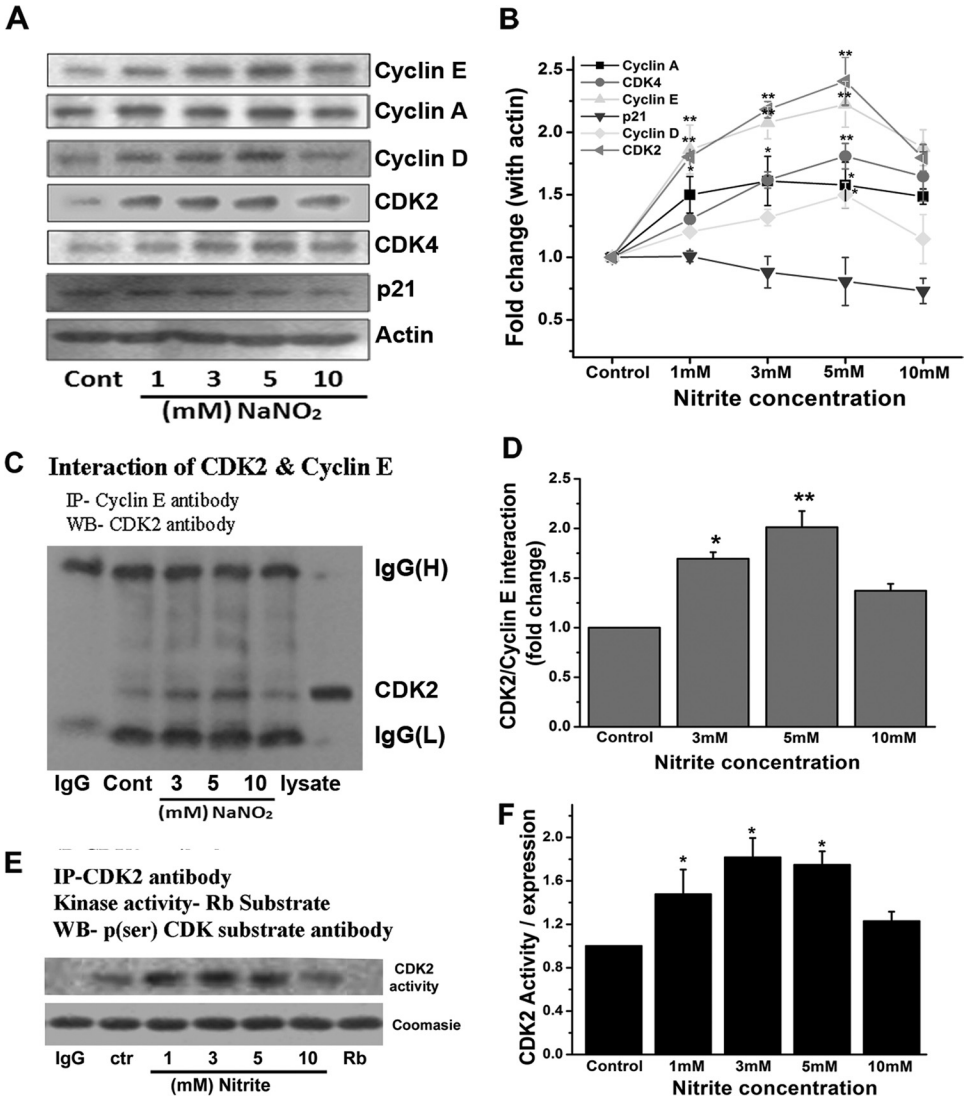

Fig. 5. Effect of sodium nitrite treatment on different cyclins/Cdks in HL-60 cells and Cdk2 involvement. A, Western blot analysis of cyclin A, cyclin D, cyclin E, Cdk2, Cdk4, and p21 after nitrite treatment. B, quantitative data of protein expression in response to nitrite treatment after normalization with actin. C and D, interaction of cyclin E and Cdk2 was explored after immunoprecipitation of cyclin E and probing with Cdk2 antibody. E, Cdk2-associated kinase activity was assessed by using Rb as substrate. F, change in Cdk2 activity with respect to its expression in nitrite-treated cells. *, $p < 0.01$; **, $p < 0.001$ in comparison with vehicle versus nitrite treatment.

up-regulation (Fig. 6), suggesting the involvement of Cdk2 in nitrite-mediated cell proliferation.

Discussion

Nitric oxide regulates cell survival, proliferation, differentiation, and apoptosis (Brookes et al., 2000; Li and Wogan, 2005; Lu et al., 2007; Kumar et al., 2010); however, NO donors have not been effectively used for the therapeutic purposes because of nonselectivity, thiol dependence, toxicity, and systemic pressor effects (Kumar et al., 2008). Consequently, the pharmacological stance on nitrite has undergone a surprising metamorphosis, from it being a vilified substance that generates carcinogenic nitrosamines to a life-saving drug (Bryan et al., 2005). In the present study, we have investigated nitrite-mediated cell cycle/proliferation mechanisms by using the neutrophilic cell line HL-60.

In the present study, sodium nitrite augmented the S phase and proliferation of HL-60 cells in a concentration-dependent manner. Nitrite induced cell proliferation in the mice intestine (Grudzinski and Law, 1998), significantly increased ischemic limb vascular density, and stimulated endothelial cell proliferation (Kumar et al., 2008). In the present study, 10 mM nitrite showed a trend of cell accumulation in the G_0/G_1 phase, as was observed previously at low sodium nitrite concentrations (up to 6.25 mM), whereas higher concentrations reduced the cell proliferation in the human gastric adenocarcinoma epithelial cell line (Sun et al., 2006). In another study on endothelial cells, the presence of nitrite (10 mM), but not nitrate, during UV irradiation of cells exerted a potent and concentration-dependent protection against apoptotic cell death, because of the photodecomposition of nitrite to generate NO (Suschek et al., 2003). BrdU incorporation confirmed the nitrite-mediated increase in S phase. The study was further extended to 72 h with 1 mM sodium nitrite, which exhibited enhanced thymidine incorporation. We did not observe apoptosis at any of the concentrations used in the present study. Oral sublethal doses of sodium nitrite (25, 50, and 100 mg/kg) transiently reduced the lymphocyte count in BALB/c mice, but significantly augmented circulating neutrophils and phagocytic activity in a dose-dependent manner (Abuharfeil et al., 2001). Later, reduction in lymphocytes was confirmed, although no effect on fibroblast proliferation was observed (Ustyugova et al., 2002). Thus nitrite-mediated effect varied with the cell type. In animals, orally administered nitrite at a daily dose of up to 74 mM for 7 days displayed neither acute toxicity nor carcinogenic activity (National

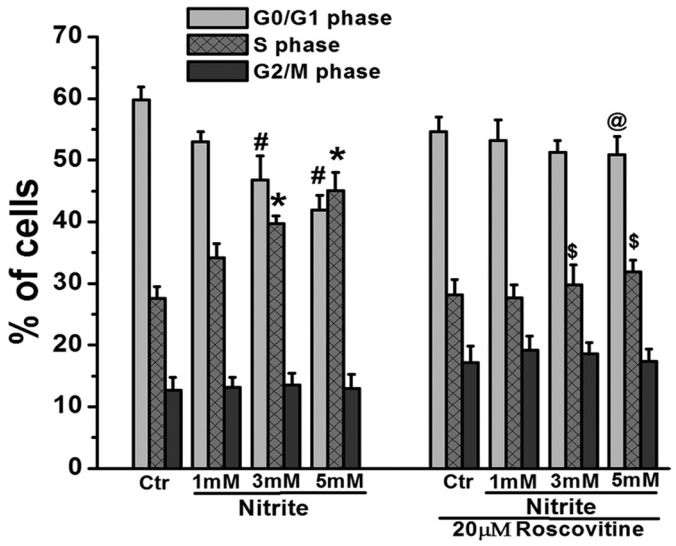

Fig. 6. Effect of roscovitine on nitrite-mediated cell cycle modulation. Roscovitine (20 μM) was added before the addition of nitrite, and its effect on cell cycle was analyzed after 24 h. Data are presented as percentage of cells. Means ± S.E.M. of five individual experiments. #, $p < 0.01$ in comparison with the G_0/G_1-phase cells in control versus nitrite-treated cells. *, $p < 0.001$ in comparison of S-phase cells in controls versus nitrite-treated cells. $, $p < 0.01$ in comparison of S-phase cells in roscovitine- versus nitrite-treated cells. @, $p < 0.001$ in comparison of G_0/G_1 cells in roscovitine- versus nitrite-treated cells.

Toxicology Program, 2001; Suschek et al., 2003). Moreover, in vitro cell culture studies with nitrite up to concentrations of 10 mM have not shown any toxic or apoptotic effects (Suschek et al., 2003; Sun et al., 2006), as was observed in the present study.

Studies on synchronized cells confirmed the proliferative effect of nitrite (Fig. 2). Research has revealed that nitrite can act as a selective NO donor, because reduction of nitrite can generate NO by several mechanisms, including, but not limited to, deoxyhemoglobin, deoxymyoglobin, xanthine oxidoreductase, and acidic environment (Cosby et al., 2003; Dezfulian et al., 2007; Kumar et al., 2008; Lundberg et al., 2008). Moreover, cytochrome *c* and nitric-oxide synthases have been found to possess nitrite reductase activity (Basu et al., 2008; Mikula et al., 2009). NO release from nitrite was also observed, suggesting the involvement of NO in nitrite-mediated cell proliferation (Fig. 3). It is noteworthy that nitrite-mediated proliferation was abolished in the presence of the NO scavenger cPTIO, and the NO donor SNP, at 10 to 100 μM also augmented the S phase of the cell cycle. The effect was redox-sensitive, because nitrite-mediated proliferative effect on HL-60 cells was abolished by 1 mM DTT, a compound commonly used to eliminate NO-mediated effects (Lu et al., 2007). GSH, an important antioxidant, which was found augmented after nitrite treatment in the present study, protects the cells from oxidative stress. GSH de novo synthesis in various cell types including endothelial, epithelial cells, and fibroblasts has been found to be augmented after exogenous addition or endogenous NO generation (Moellering et al., 1999; Lu et al., 2007). The present study, however, did not explore the mechanisms involved in the release of NO from sodium nitrite.

The present study also suggests ROS involvement because nitrite-treated cells exhibited augmentation in intracellular ROS, and S-phase augmentation was rescued with ROS scavenger/inhibitor. Moreover, mitochondrial function blockers prevented the nitrite-induced S phase (Fig. 4C). A central role of mitochondria has been identified in cell cycle regulation (Mitra et al., 2009). NO is a well known modulator of cytochrome *c* oxidase activity and mitochondrial respiration (Li and Wogan, 2005). Moreover, disruption of complex I retards the G_1- to S- phase transition through ROS, which have independently been found to modulate cell cycle progression (Owusu-Ansah et al., 2008). Menon et al. (2003) have shown that a transient increase in the pro-oxidant levels push the cells for an early transit from G_1 into the S phase. NAC, an antioxidant, arrested mouse embryonic fibroblasts, hepatic stellate cells, and vascular smooth muscle cells in G_1 phase and inhibited their proliferation (Menon et al., 2003; Menon and Goswami, 2007). Growth factors also stimulate a rapid increase in intracellular ROS levels and associated cell signaling (Sattler et al., 1999; Felty et al., 2005). Low concentrations of oxidant and NO donors stimulated the proliferation of human endothelial cells in vitro (Luczak et al., 2004). Estrogen-induced cell proliferation was reduced by NAC and catalase; moreover, the mitochondrial inhibitors rotenone and antimycin also blocked the estrogen-induced G_1- to S-phase transition (Felty et al., 2005). Indeed, both free radical and mitochondrial inhibitors significantly reduced ROS to basal level in nitrite-treated cells (data not shown). Data obtained thus suggest the nitrite-mediated induction of S phase in HL-60 cells also depended on mitochondrial ROS. Moreover, we did not observe any significant change in HL-60 differentiation as assessed by cell surface markers for neutrophilic (CD11b) and monocytic (CD14) cells (data not shown).

Further, cell cycle regulators were explored to assess the molecular control in nitrite-mediated cell cycle modulation. Appropriate temporal activation of cyclin E/Cdk2 is required for the progression through G_1 to S phase, whereas p21/Cdk2 interaction inhibits cell cycle progression (Johnson and Schneider-Broussard, 1998). Expression of various cyclins and Cdks was augmented in the nitrite (1–5 mM)-treated cells, specifically Cdk2 and cyclin E expression and interaction were enhanced, leading to an increase in Cdk2 kinase activity. Cells treated with 10 mM nitrite exhibited down-regulation of cyclins/Cdks, and cyclin E/Cdk2 interaction and Cdk2 activity were also significantly reduced. Roscovitine, an inhibitor of Cdks with more specificity for Cdk2, prevented nitrite-mediated cell proliferation. Thus, nitrite seems to modulate HL-60 cell proliferation through an increase in cyclin E/Cdk2 interaction and Cdk2 activity.

The nitrite anion, which was initially thought to be biologically inactive, is now considered to be a biochemical reservoir of NO. Nitrite is ubiquitously present in biological fluids such as blood and sweat and can be augmented substantially with dietary additives. The nitrite concentration used in the present study is much more than the amount reported in plasma, but it corresponds to concentrations used in various studies (Abuharfeil et al., 2001; Suschek et al., 2003; Sun et al., 2006) and has also been used for dietary/therapeutic purposes (Bryan et al., 2008). There have been concerns about the effects of dietary nitrite on human health because nitrite can promote the generation of potentially carcinogenic nitrosamines (Tannenbaum et al., 1976). However, the link between nitrite in human cancer has yet to be established (Lundberg and Weitzberg, 2009). The present study, how-

ever, cautions about nitrite-mediated cell proliferation, before initiating its use as therapeutics in the ischemic insult or inflammatory conditions. It will be interesting to see the beneficial effect of nitrite therapy in the infective conditions where increase in neutrophil count might be helpful in counteracting pathogenic insult. The present study thus warrants research in these directions.

The present study thus demonstrates nitrite-mediated cell proliferation of the human neutrophilic HL-60 cell line via the induction of S phase of the cell cycle. The proliferative effect seems to be redox-sensitive and dependent on NO release from nitrite. Furthermore, nitrite augmented the mitochondrial-dependent intracellular ROS and its inhibition rescued nitrite-mediated effect. Up-regulation of cell cycle regulators, efficient Cdk2/cyclin E interaction, and augmented Cdk2 activity were observed in nitrite-treated cells. Indeed, the proliferative effect of nitrite was blocked by roscovitine, a Cdk2 inhibitor. The present study for the first time demonstrated the induction of S phase and the involvement of Cdk2 in nitrite-mediated HL-60 cell cycle progression and proliferation, warranting detailed investigations before its clinical use in various pathological conditions.

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Authorship Contributions

Participated in research design: Dikshit.

Conducted experiments: Kumar.

Contributed new reagents or analytic tools: Barthwal and Dikshit.

Performed data analysis: Kumar, Barthwal, and Dikshit.

Wrote or contributed to the writing of the manuscript: Kumar and Dikshit.

Other: Dikshit acquired funding for the research.

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