Nitrite mediated modulation of HL-60 cell cycle and proliferation:

Involvement of Cdk2 activation

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(S.K., M.K.B., M.D.)
Running Title: Nitrite and cell cycle regulation

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Number of text pages: 30
Number of tables: 1
Number of figures: 6
Number of references: 40
Number of words in the Abstract: 248
Number of words in the Introduction: 711
Number of words in the Discussion: 1393

Abbreviations: BrdU, 5-bromo-2-deoxyuridine; Cdk, cyclin dependent kinase; DTT, Dithiothreitol; NAC, N-acetyl-L-cysteine; NO, Nitric oxide; ROS, reactive oxygen species.

Recommended section: Cellular and molecular
Abstract

Recent research suggests vivid possibility of 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 (0.5-10mM) on the proliferation of neutrophilic cell line HL-60 and have also explored the underlying mechanism. Treatment of HL-60 cells with sodium nitrite (0.5-5mM) led to an increase in the cell proliferation, which was confirmed by cell cycle analysis, BrdU and thymidine incorporation, while cells accumulated in G0/G1 phase following treatment with 10mM nitrite. Experiments on the synchronized cells exhibited similar effect, which seems to be NO dependent, as cPTIO abolished nitrite mediated proliferative effect. Moreover, NO donor SNP at micromolar concentration also exhibited similar effects. Nitrite induced augmentation in S phase and intracellular ROS generation was prevented by ROS scavenger/inhibitors. Moreover, mitochondrial blockers, rotenone and antimycin A also reduced nitrite mediated cell proliferation. Assessment of cell cycle regulators Cdk2, Cdk4, cyclin A, cyclin D, cyclin E, p21 suggested augmentation in the expression and interaction of Cdk2/cyclin E, Cdk2 activity, while p21 was down-regulated. Indeed proliferative effect of nitrite was blocked by roscovitine, a Cdk2 inhibitor. The results obtained thus 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 following 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 curing foods to impart pleasant colour to meat and to prevent botulism (Gladwin et al., 2005; Butler and Feelisch, 2008). While under certain conditions, nitrites mediate formation of nitrosamines, which are potent carcinogens (Tannenbaum et al., 1976). Although, recent studies have found no direct correlation of nitrite mediated formation of N-nitrosamines with carcinogenesis (Lundberg and Weitzberg, 2009). Moreover, recent research has also suggested role of nitrite in important physiological and patho-physiological 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 catalyse 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 signalling molecule under physiological as well as ischemic conditions (Bryan et al., 2005). Nitrite also serves a diagnostic marker and plays a role of potential therapeutic agent in offering protection against myocardial and liver ischemia reperfusion induced injury (Webb et al., 2004; Bryan et al., 2007). Moreover, use of chronic dietary nitrite supplementation (50 mg/L) in the drinking water for one week, restored NO homeostasis in eNOS−/− mice and protected against I/R 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 following 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), interestingly growth factors stimulate rapid increase in intracellular ROS that modulate signalling involved in the cell growth (Sattler et al., 1999; Felty et al., 2005). The G1 to S phase transition in fibroblasts depends on the intracellular redox status (Menon et al., 2003). Recently, mitochondria has been found to play a central role in the cell cycle regulation (Mitra et al., 2009). Moreover, disruption of the mitochondrial electron transport chain specifically retards the G1 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).

Recently, we demonstrated a biphasic regulation of HL-60 cell cycle and proliferation by NO that were mediated by Cdk2 nitrosylation, while apoptosis was due to the loss of mitochondrial potential (Kumar et al., 2010). Augmentation of neutrophils 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 (0.5-5mM) augmented HL-60 cell
proliferation and cell cycle S phase in a concentration dependent manner, while treatment with 10mM 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 pre-treatment inhibited proliferative effect of nitrite. The present study thus explores nitrite mediated cell cycle regulation of HL-60 cells and proposes the involved putative mechanisms.
Materials and methods

Chemicals and reagent
Sodium nitrite, sodium nitrate, 7-aminoactinomycin D (7-AAD), N-acetyl-L-cysteine (NAC; ROS scavenger), antimycin A (Complex III Inhibitor), carboxy-1H-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 (La Jolla, CA). The annexin V FITC kit and BrdU FITC Kit were purchased from BD Biosciences (San Diego, CA).

Cell culture and nitrite treatment
HL-60 cells (ATCC) were grown in RPMI-1640 containing NaHCO₃, 2mM glutamine, 10% (v/v) FBS, 100 units/ml penicillin and 100µg/ml streptomycin at 37°C in a 5% CO₂ atmosphere (Kumar et al., 2010). Cells were seeded at a concentration of 2x10⁵ cells/ml and were maintained for logarithmic growth by passaging them every 2–3 days and incubated for 1–3 days as per requirement of experiment with sodium nitrite at various concentrations (0.5-10mM). To explore the putative mechanism, cells were pre-treated with various interventions/vehicle like cPTIO, a NO scavenger, Dithiothreitol (DTT), reactive oxygen species (ROS) inhibitors, mitochondrial blockers, (rotenone and antimycin A) and Cdk2 inhibitor (roscovitine) prior to nitrite treatment as mentioned in figure legends.

Cell viability assessment
Vehicle or nitrite treated HL-60 cells (1x10⁵ cells/ml) in the culture medium were directly incubated with propidium iodide (PI, 5µg/ml in PBS) 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 were subsequently analyzed using Cell Quest 5.2 program (FACS Calibur, Becton Dickinson, USA). Annexin V labelling of cells was also performed using annexin V FITC kit (Becton Dickinson) (Kumar et al., 2010).

**Cell cycle analysis**

DNA staining was performed (Krishan, 1975) in control/vehicle, nitrite, SNP or in the cells treated with various interventions as mentioned in the figure legends. Briefly, HL-60 cells (1x10^5) were centrifuged at 150g for 5 min and the pelleted cells were re-suspended in hypotonic propidium iodide solution (50µg/ml with 0.03% NP-40 in 0.1% sodium citrate). Samples were acquired after 10-20 min of staining at 4°C. DNA content in these cells was assessed using a flow cytometer (FACS Calibur, Becton Dickinson) and Cell Quest program, while cell-phase distribution was analyzed by Modfit 3.0 software (Verity Software, Topsham).

**Cell synchronization**

L-Mimosine was used for cell synchronization at G0 phase as described earlier (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 (50µg/ml with 0.03% NP-40 in 0.1% sodium citrate) and analyzed by FACS calibur (Becton Dickinson). 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.
**3H-Thymidine incorporation assay**

HL-60 cells (1x 10^5 cells) were cultured in 96-well plates in RPMI-1640 with 10% FBS, sodium nitrite was added at 1mM concentration. One µCi of 3H thymidine was added to the plates in last 24 h and cultured in 5% CO₂ at 37°C. After 24, 48 and 72 h, the cells were harvested on a semiautomated cell harvester (Biotron Healthcare, USA) and the radioactivity was measured with a scintillation counter (Wallac, USA). All the experiments were performed in triplicate.

**BrdU Incorporation assay**

DNA synthesis during S phase was assessed by measuring the incorporation of 5-bromo-2-deoxyuridine (BrdU) into DNA using a cell proliferation kit (BrdU FITC Kit; BD Biosciences) according to the manufacturer's instructions. Briefly, cells were pulsed with 10µM BrdU for 30 min in culture followed by fixation with BD Cytofix/Cytoplasm buffer, permeabilization by BD Cytoperm plus buffer, DNAase treatment and finally detection with anti BrdU- FITC antibody. DNA was simultaneously stained with 7-AAD. Cells were analysed on 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). NO donor, SNP was used as standard. DAF (10µM) 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 Caryecclipse).
Western blot analysis

Cells were lysed with protein extraction buffer; lysis buffer (Tris 20 mM; pH 7.5 containing 1% Triton X-100, 150 mM NaCl, 1 mM Na3VO4, 1 mM NaF and protease inhibitors). Protein concentrations were measured by BCA protein assay reagent kit (Pierce, Illinois, USA). Samples containing equal amounts of protein (40µg) were run on 10-12% SDS-PAGE and transferred to PVDF membranes (Amersham Biosciences, Sweden). Blots were probed with antibodies specific for cyclin A, cyclin E, cyclin D, p21, Cdk2, Cdk4, (Cell Signalling Technology, Boston, USA), or actin (Sigma-Aldrich) at 1:1000 dilution in TBST [Tris 25mM, NaCl 150mM, tween 20 (0.1%)]. Further, appropriate horse radish peroxidase-conjugated secondary antibodies (Sigma-Aldrich, St. Louis, MO) were used. Chemiluminescent visualization of proteins was done by using ECL-plus (GE Healthcare, Uppsala, Sweden) (Kumar et al., 2010).

Cdk2 kinase assay

In vitro Cdk2 kinase activity was measure 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 PMSF, 1 mM NaF, 20 mM sodium pyrophosphate, 1 mM sodium vanadate, 10 µg/ml each of leupeptin, aprotinin and pepstatin) on ice for 30 min. Total 200 µg of protein lysate, after pre-clearing with equilibrated protein A beads were incubated overnight with 1 µg of mouse anti-Cdk2 IgG (BD Biosciences) at 4°C followed by immuno precipitation 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 MgCl2 and 1 mM DTT). The Cdk2 kinase reaction were performed at 30°C for 30 min in kinase buffer containing 5µg Retinoblastoma (RB) protein [(aa 773-928) Millipore, USA] as substrate, and 100 µM ATP. The reaction was stopped by adding 2x Laemmlil buffer and
boiled for 5 min. Phosphorylation of RB was analyzed on 10% SDS-PAGE followed by Western blotting with p(Ser) Cdk substrate antibody (Cell Signalling Technology, Boston, MA, USA), which detected phosphorylation of RB protein.

**Assessment of intracellular glutathione**

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

**Statistical analysis**

Data have been represented as Mean ± SEM, of at least 3-5 independent experiments and were analyzed by one way ANOVA test followed by Newman-Keuls post analysis. Student’s t-test analysis was also used to compare the Control Vs treated as has been 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 (0.5-10mM) for 24 h augmented S phase of cell cycle significantly (Fig. 1a,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 has been illustrated in Fig. 1b. To ensure the specificity of nitrite, sodium nitrate was used, which had no effect on 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 1mM nitrite for 24 to 72 h exhibited proliferation, as demonstrated by an increase in 3H-thymidine incorporation (Fig. 1d). Under the experimental conditions used there was no loss in the cell viability (PI permeable) at all the concentrations of nitrite (Fig. 1e). Moreover, Annexin V labelling, revealed only 4-6% Annexin V positive cells in control and nitrite treated cells. However, treatment of cells with 10mM sodium nitrite showed a trend of cells accumulation in G0/G1 phase, even though there was significantly more number of cells in S-phase in comparison to the vehicle treated control cells (Fig 1a). In order to establish the proliferative effect of sodium nitrite on HL-60 cells, further experiments were performed at 1-5mM concentration.

Effect of nitrite on synchronized cells

Further synchronized cells were also used to validate nitrite mediated modulation of cell cycle. HL-60 cells were synchronized in G1 phase with mimosine (0.5mM) for 18 h to confirm the proliferative effect (Fig. 2 a). Mimosine mediated block was reversible as removal of mimosine led to the re-entry of cells into S and G2M phase (Fig. 2 b), but substantial number of cells died. Addition of nitrite to G1 synchronized cells demonstrated
entry of more cells in S phase after 24 h treatment using nitrite (Fig 2 c), which was also confirmed by BrdU incorporation (Fig 2 d).

Role of NO in mediating effect of nitrite

Nitrite mediated effects under both physiological and patho-physiological conditions have been implicated to be mostly due to reduction of nitrite to NO (Webb et al., 2004; Bryan et al., 2008; Butler and Feelisch, 2008), we therefore explored involvement of NO in nitrite mediated cell cycle modulation. The increase in S phase by nitrite treatment was abolished in the presence of 300µM NO scavenger, cPTIO, suggesting that NO could be a mediator of nitrite effect (Table 1). Addition of slow NO releaser, sodium nitroprusside (SNP), also exhibited proliferative effect on HL-60 cells at 10-100µM concentration (Fig. 3 a). To investigate nitrite mediated NO release, we monitored kinetics of diamino-fluorescein (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 release of NO (Fig. 3 b). The effect of nitrite on cell cycle seems to be due to NO, which was also confirmed by pre-treatment with DTT (1mM), which inhibited NO mediated events (Fig. 3 d). Furthermore GSH content was also found to be significantly augmented in nitrite treated cells (Fig. 3 c), suggesting the redox regulation in nitrite mediated proliferation.

Role of ROS and mitochondria in modulating nitrite mediated cell cycle

ROS regulate cellular signalling 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 (0.5-5mM) treated cells in a concentration dependent manner as was assessed by DCF (a broad range ROS detector) and DHE (a superoxide specific dye) (Fig. 4 a,b). Furthermore, free radical scavenger N-acetyl-L-cysteine (NAC) or inhibitor DPI pre-treatment significantly reduced
nitrite mediated augmentation in S phase and cell proliferation to the vehicle treated control level (Fig. 4 c). Recently, mitochondria were identified to play a key role during S phase transition, which are also 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. 4 c). Though 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 induced cell cycle S phase of HL-60 cells was also dependent on mitochondria mediated ROS generation.

**Effect of nitrite on cyclins/Cdk5 expression and Involvement of Cdk2**

Expression of various cyclins/Cdk5s, which regulate the progression of cells from G1 to S phase, such as cyclin A, cyclin D, cyclin E, Cdk2, Cdk4 and p21 were monitored (Fig. 5 a,b). Western blotting of these proteins following treatment of sodium nitrite, exhibited significant enhancement of cyclin A, cyclin D, cyclin E, Cdk2 and Cdk4 expression at 1-5 mM nitrite, while expression of cell cycle inhibitor p21 was reduced. These results overall represent more active cell cycle machinery in the proliferating cells following treatment with 1-5 mM nitrite. However treatment with 10mM 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 the progression through the G1 and S entry, while p21/Cdk2 interaction inhibits cell cycle progression. Moreover, Cdk2 and cyclin E interaction was also investigated and was found to be augmented in upto 5mM sodium nitrite treated cells (Fig. 5 c,d). Further, Cdk2 kinase activity was measured by using RB protein, a substrate of Cdk2,
which was augmented at 1 to 5mM nitrite concentration (Fig. 5 e). Increase in Cdk2 activity was not dependent on enhanced Cdk2 expression as evident by activity/expression ratio (Fig. 5 f). However, sodium nitrite (10mM) rescue cyclin E/Cdk2 interaction and Cdk2 kinase activity as well, thus suggesting cytostasis and no cell death was observed.

Furthermore, most commonly used purine analog, roscovitine a potent and selective inhibitor of cyclin-dependent kinases (Cdk), having high specificity towards Cdk2, was used to explore the role of Cdk2 in nitrite mediated cell cycle regulation. Out of the three doses of 10, 20 and 50µM, 20µM of Roscovitine used in the present study, 20µM was the optimal concentration, while 50µM induced cell death (data not shown). Roscovitine (20µM) prevented nitrite (1-5mM) mediated S phase up-regulation (Fig. 6), suggesting involvement of Cdk2 in nitrite mediated cell proliferation.
Discussion

Nitric oxide, regulates cell survival, proliferation, differentiation as well as apoptosis (Brookes et al., 2000; Li and Wogan, 2005; Lu et al., 2007; Kumar et al., 2010), however NO donors have not been effectively utilised for the therapeutic purposes due to non-selectivity, thiol dependency, toxicity, and systemic pressor effects (Kumar et al., 2008). Consequently pharmacological stance on nitrite, has undergone a surprising metamorphosis, from 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 mechanism(s) using a neutrophilic cell line HL-60.

In the present study, sodium nitrite augmented 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, 10mM nitrite showed a trend of cells accumulation in G0/G1 phase, as was previously observed at low sodium nitrite concentrations (up to 6.25 mM), while higher concentrations reduced the cell proliferation in human gastric adenocarcinoma epithelial cell line (Sun et al., 2006). In yet another study on endothelial cells, presence of nitrite (10mM) but not nitrate, during UV-irradiation of cells exerted a potent and concentration-dependent protection against apoptotic cell death, due to 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 1mM sodium nitrite, which exhibited enhanced thymidine incorporation. We did not observe apoptosis at any of the concentration used in the present study. Oral sub-lethal doses of sodium nitrite (25, 50 and 100 mg/kg) though transiently reduced the lymphocyte count in Balb/c mice, but significantly augmented circulating neutrophils and the phagocytic activity in a dose dependent manner (Abuharfeil et al., 2001).
Later, reduction in lymphocytes was confirmed, while no effect on the fibroblasts 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 (NTP, 2001; Suschek et al., 2003). Moreover, in vitro cell culture studies with nitrite up to 10 mM concentrations have not shown any toxic or apoptotic effects (Suschek et al., 2003; Sun et al., 2006), as was also observed by us in the present study.

Studies on synchronised cells confirmed the proliferative effect of nitrite (Fig. 2). Recent research revealed that nitrite can act as a selective NO donor, as 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 involvement of NO in nitrite mediated cell proliferation (Fig. 3). Interestingly, nitrite mediated proliferation was abolished in the presence of NO scavenger cPTIO, and NO donor SNP, at 10-100µM also augmented S phase of cell cycle. The effect was redox sensitive, since nitrite mediated proliferative effect on HL-60 cells was abolished by 1 mM DTT, a compound commonly used to eliminate NO mediated effect (Lu et al., 2007). GSH, an important antioxidant, which was found augmented following 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 following exogenous addition or endogenous NO generation (Moellering et al., 1999; Lu et al., 2007). The present study however has not explored the mechanisms involved in the release of NO from sodium nitrite.
The present study also suggests ROS involvement as nitrite treated cells exhibited augmentation in the intracellular ROS and S phase augmentation was rescued with ROS scavenger/inhibitor. Moreover, mitochondrial function blockers prevented the nitrite induced S phase (Fig. 4c). Recently, a central role of mitochondria has been identified in the cell cycle regulation (Mitra et al., 2009). NO is well known modulator of cytochrome c oxidase activity and mitochondrial respiration (Li and Wogan, 2005). Moreover, disruption of complex I retards the cell cycle G1 to S 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 G1 into the S phase. NAC, an antioxidant arrested the mouse embryonic fibroblasts, hepatic stellate cells and vascular smooth muscle cells in G1 phase and inhibited their proliferation (Menon et al., 2003; Menon and Goswami, 2007). Growth factors also stimulate a rapid increase in the intracellular ROS levels and associated cell signalling (Sattler et al., 1999; Felty et al., 2005). Low concentration of oxidant and NO donors stimulated proliferation of human endothelial cells in vitro (Luczak et al., 2004). Estrogen-induced cell proliferation was reduced by NAC and catalase, moreover mitochondrial inhibitors rotenone and antimycin also blocked estrogen-induced G1 to S 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 nitrite mediated induction of S phase in HL-60 cells was also dependent 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 the nitrite mediated cell cycle modulation. Appropriate temporal activation of cyclin E/Cdk2 is required for the progression through G1 to S phase, while 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 the Cdk2 kinase activity. While, cells treated with 10mM nitrite, exhibited down regulation of cyclins/Cdks, indeed cyclin E/Cdk2 interaction and Cdk2 activity was 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 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 augment substantially with dietary additives. Although the nitrite concentration used in the present study is much more than the amount reported in the 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 recently 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, link between nitrite in human cancer is yet to be established (Lundberg and Weitzberg, 2009). The present study however cautions about the nitrite mediated cell proliferation, prior to initiate its use as therapeutics in the ischemic insult or inflammatory conditions. It will however be interesting to see the beneficial effect of nitrite therapy in the infective conditions where increase in neutrophil count might be helpful in counteracting the pathogenic insult. The present study thus warrants research in these directions.

The present study thus demonstrates nitrite mediated cell proliferation of 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. Further, 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 was observed in nitrite treated cells. Indeed proliferative effect of nitrite was blocked by roscovitine, a Cdk2 inhibitor. The present study for the first time demonstrated induction of S phase and involvement of Cdk2 in the nitrite mediated HL-60 cell cycle progression and proliferation, warranting detailed investigations prior to its clinical use in various pathological conditions.
Acknowledgements

The authors gratefully acknowledge the technical help provided by Mr. CP Pandey, Mrs. M Chaturvedi and Mr. AL Vishwakarma. This is CDRI communication No. 7926.

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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Footnotes

**Grant Support:** The study was supported by a financial grant to M Dikshit from the Department of Biotechnology, India, and an award of research fellowship to SK from the Council of Scientific and Industrial Research, India is acknowledged.

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Figure legends

Figure 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 (50µg/ml with 0.03% NP-40 in 0.1% sodium citrate), cell cycle-phase distribution was analyzed by Modfit software (Verity Software, Topsham). (B) Quantitative data of cell cycle distribution in control and nitrite treated cells (* p <0.01,** p <0.001 in comparison to controls S phase and # p <0.01 in comparison to G1 phase of control). (C) Dot plot exhibiting BrdU incorporation in control and nitrite treated cells (0.5-10 mM) after 24 h. Y-axis shows significantly more cells in S phase (DNA synthesis) after nitrite treatment. (D) HL-60 cells were cultured in 96-well plates with nitrite (1mM). 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. 3H thymidine (1µCi) was added to the cells in last 24 h of nitrite treatment in 5% CO2 at 37°C. Data have been presented as Means ± SEM of at least 5 experiments, * p <0.01 in comparison to the control. (E) Cell viability was analysed by quantifying the PI impermeable cells in control and nitrite treated cells by using flow cytometry as described in material and methods.

Figure 2. Effect of sodium nitrite on synchronized HL-60 cells. (A) Cells were synchronized in G1 phase with mimosine (0.1-0.5mM) for 18-24 h (B) This block was reversible, as cells cultured for 24 h without mimosine reverted to proliferation (C) Synchronized cells treated with nitrite (1-5mM) 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 30min as described in materials and methods. (**p <0.001 in comparison to controls vs nitrite treatment).
Figure 3. Effect of SNP on HL-60 cells, nitrite mediated effect on GSH and modulatory effect of DTT. (A) Cell cycle-phase distribution after 10-100μM SNP treatment of HL-60 cells for 24 h. (B) Nitrite mediated NO release in present experimental conditions was assessed by DAF fluorescence kinetics, NO donor SNP was used as reference NO releaser. (C) GSH content after treatment of HL-60 cells at different concentration of nitrite (1-5mM). (Data are presented of 3 individual experiments as fold change in fluorescence of nitrite treated vs controls, *p <0.01 in comparison to controls cells vs nitrite treatment). (D) Treatment of cells with 1mM DTT for 30 min prior to nitrite addition (1-5mM) and cell cycle was analysed. (Data are presented as % of cells, Means ± SEM of 5 individual experiments, *p <0.01 in comparison to the G0G1 phase cells in controls vs nitrite treated cells, *p <0.001 in comparison to S phase cells in controls vs nitrite treatment, @, $p <0.01 in comparison of DTT vs nitrite treatment).

Figure 4. Involvement of ROS and mitochondria in nitrite mediated effect. (A) 1x10^6 cells were incubated with 10μM DCF-DA (A) or 10μM DHE (B) for 30 min at 37οC and 10,000 cells from each tube were acquired and analyzed by a Flow cytometer using Cell Quest program. * p <0.01, ** p <0.001 in comparison to vehicle vs nitrite treatment. (C) Cell cycle-S phase was analyzed by Modfit software (Verity Software, Topsham) in cells pre-incubated with NAC, DPI, rotenone or antimycin A, before nitrite treatment. (* p <0.01, ** p <0.001 in comparison to controls S phase and # p <0.01 in comparison to nitrite treated cells).

Figure 5. Effect of sodium nitrite treatment on different Cyclins/Cdks in HL-60 cells and Cdk2 involvement. (A) Western blot analysis of cyclin A, D, E, Cdk2, Cdk4 and p21 after nitrite treatment (B) Quantitative data of protein expression in response to nitrite
treatment after normalization with actin (C,D) Interaction of cyclin E and Cdk2 was explored after IP 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 to vehicle vs nitrite treatment.

Figure 6. Effect of roscovitine on nitrite mediated cell cycle modulation. Roscovitine (20µM) was added prior to the addition of nitrite and its effect was analysed after 24 h on cell cycle. (Data are presented as % of cells, Means ± SEM of 5 individual experiments, * p <0.01 in comparison to the G0G1 phase cells in control vs nitrite treated cells, *p <0.001 in comparison to S phase cells in controls vs nitrite treated cells, $ p <0.01 in comparison of S phase cells in roscovitine vs nitrite treated cells and @p <0.001 in comparison of G0G1 cells in roscovitine vs nitrite treated cells).
Table 1: Effect of c-PTIO on nitrite mediated cell cycle modulation.

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Cells were treated with 300µM c-PTIO for 30 min prior to nitrite addition (1-5mM). Distribution of cells in various phases of Cell cycle was analysed by Modfit software (Verity Software, Topsham). (Data have been presented as % of cells, Means ± SEM of 5 individual experiments, * p <0.01, **p <0.001 in comparison to S phase cells in control vs post nitrite treatment, @p <0.01 in comparison of nitrite vs c-PTIO treatment).
Figure 1

A

B

C

D

E

\[ \text{JPET Fast Forward. Published on March 16, 2011 as DOI: 10.1124/jpet.110.177444} \]

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