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Journal of Cellular Biochemistry 112:2942–2952 (2011)

Journal of Cellular Biochemistry

Hyperglycemia Regulates MDR-1, Drug Accumulation and ROS Levels Causing Increased Toxicity of Carboplatin and 5-Fluorouracil in MCF-7 Cells

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ABSTRACT

There is constant increase in number of diabetic cases thereby giving it status of a serious epidemic. Diabetes increases the risk of occurrence of several cancers including breast cancer and may also have a serious impact on the outcome of cancer treatment. In the present study we investigated effect of hyperglycemia on cytotoxic efficacy of carboplatin and 5-fluorouracil in MCF-7 cells. MCF-7 cells were grown in 5.5 or 25 mM glucose chronically. We show that hyperglycemia favors proliferation of MCF-7 cells and increases expression of cell cycle regulatory proteins cyclin E and cdk-2. Hyperglycemia enhances cytotoxicity of carboplatin and 5-fluorouracil in MCF-7 cells by approximately 30% and decreases their IC50 by 1.5- and 1.3-folds, respectively. Hyperglycemia reduces expression of P-glycoprotein and promotes cell killing by increasing drug accumulation. Treatment with 40 μM verapamil, an inhibitor of P-gp activity specifically increases killing of MCF-7 cells cultured in 5.5 mM glucose. Further, this effect is synergized by elevated reactive oxygen species and treatment with *N*-Acetylcysteine, an inhibitor of ROS, increases survival by 30 and 18% in carboplatin- and 5-fluorouracil-treated cells cultured in high glucose, respectively. Cytotoxicity of these drugs is associated with reduced activation of Akt and decreased transcriptional activation of NF-κB. In conclusion, hyperglycemia potentiates cytotoxicity of drugs by reducing P-gp expression and, increased ROS levels may partially or completely contribute to enhanced toxicity. J. Cell. Biochem. 112: 2942–2952, 2011.

KEY WORDS: HYPERGLYCEMIA; BREAST CANCER; DNA DAMAGING DRUGS; CYTOTOXICITY

Magnitude of diabetes and cancer is increasing at an alarming rate all over the world. Diabetes and cancer have been projected as frequently encountered comorbid situations. Therefore, growing epidemic of diabetes is likely to have a significant impact on the health care in cancer patients [Singer, 2007]. Diabetic hyperglycemia may negatively impact both cancer risk and outcome of treatment. Glucose mainly regulates insulin and glucagon, two hormones important for controlling its metabolism. Therefore, it is particularly difficult to discriminate the effects of glucose on gene transcription from these two hormones [Lefrancois-Martinez et al., 1995]. However, establishment of cell lines has overridden this barrier and in several studies these have been successfully used to study the direct effect of glucose on proliferation of mammalian cells [Lefrancois-Martinez et al., 1995; Turturro et al., 2007a; Turturro et al., 2007b]. Only limited literature is available on the effect of diabetes related complications towards the outcome of cancer chemotherapy. Earlier studies have suggested that hyperglycemia may enhance the cytotoxicity of drugs in murine models of glioma and melanoma [Schem et al., 1995; Canter et al., 2004]. This observation was supported by the report from Kung et al. [1963], demonstrating potentiation of anti-tumor effect of 5-fluorouracil (5-FU) by glucose in rats with Flexner jobling carcinoma. Recently, it has also been reported that hyperglycemia enhances the cytotoxicity of paclitaxel in MDA-MB 231 cells [Turturro et al., 2007b]. Sadoff [1998] proposed that patients whose diabetes is poorly controlled are at increased risk for severe 5-FU cytotoxicity.

The effectiveness of any chemotherapeutic drug is largely dependent on its accumulation and retention by cells. The reduced retention of chemotherapeutic drug in cancer cells is primarily due

Abbreviations: 5-FU, 5-fluorouracil; Carb, carboplatin; CM-H2DCFDA, chloromethyl-2′,7′-dichlorofluorescein diacetate; MCF-HG, MCF-7 cells cultured in DMEM with 25 mM glucose; MCF-MNTL, MCF-7 cells cultured in DMEM with 5.5 mM glucose + 19.5 mM mannitol; MCF-NG, MCF-7 cells cultured in DMEM with 5.5 mM glucose; MTT, methylthiazole tetrazolium; P-gp, P-glycoprotein; ROS, reactive oxygen species; VPL, verapamil.

Competing interests: The authors declare that they have no competing interests.

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Received 20 August 2010; Accepted 25 May 2011 • DOI 10.1002/jcb.23210 • © 2011 Wiley-Liss, Inc.

Published online 31 May 2011 in Wiley Online Library (wileyonlinelibrary.com).

to overexpression of P-glycoprotein (P-gp) [Hamada and Tsuruo, 1988; Horio et al., 1988] and its expression is significantly elevated in drug-resistant tumors of colon, kidney, and liver [Goldstein et al., 1989]. Also, cytotoxicity of various anticancer agents has been attributed to increased intracellular reactive oxygen species (ROS) levels [Kotamraju et al., 2000; Ravi and Das, 2004]. In response to hyperglycemia, ROS increases and antioxidant defense capabilities are diminished [Araki and Nishikawa, 2004]. A moderate level of intracellular ROS is thought to be essential for cellular proliferation [McCord, 1995]. On the other hand, exposure of cancer cells to ROS generating anticancer agents exhausts the cellular antioxidant capacity and ROS level beyond a threshold causes apoptosis [Kong et al., 2000].

In the present study, we observed an enhanced cytotoxicity of DNA damaging drugs carboplatin and 5-FU on MCF-7 cells chronically cultured under hyperglycemic conditions. Interestingly, we observed a reduced expression of P-gp and enhanced accumulation of drug together with increase in basal level of ROS, in these cells. It is therefore likely that elevated ROS synergizes with enhanced drug accumulation to potentiate the cytotoxicity of carboplatin and 5-FU.

MATERIALS AND METHODS

DRUGS AND CHEMICALS

Carboplatin and 5-fluorouracil (5-FU) were purchased from Sigma, St. Louis and dissolved in sterile water to prepare a stock of 25 and 50 mM, respectively. *N*-Acetylcysteine (NAC) and verapamil (VPL) (Sigma, St. Louis) were dissolved in sterile water to prepare a 100 mM stock (St. Louis). Radiolabeled 5-fluorouracil (^{14}C-5-FU) and tritiated thymidine were purchased from American Radio Labeled Chemicals (ARC) Inc., St. Louis.

CELLS AND CULTURE CONDITIONS

Human breast cancer cell line MCF-7 and hepatoma cell line HepG2 were obtained from American Type Culture Collection (ATCC) Manassas, VA and maintained in our in-house cell repository. MCF-7 and HepG2 cells were routinely cultured over a period of 3 months in Dulbecco's modified eagles medium (DMEM) containing either physiologically normoglycemic (5.5 mM) or hyperglycemic (25 mM) glucose supplemented with 10% heat inactivated fetal bovine serum (Hyclone, Utah), penicillin (100 U/ml) and streptomycin (100 μg/ml) (Invitrogen Corporation, CA) at 37°C with 5% CO_2. As a control for osmolarity cells were chronically cultured in DMEM (5.5 mM glucose) supplemented with 19.5 mM mannitol (Sigma, St. Louis). Hereafter, MCF-7 cells cultured in 5.5, 25, and 5.5 mM glucose supplemented with 19.5 mM mannitol will be referred as MCF-NG, MCF-HG, and MCF-MNTL, respectively.

CELL PROLIFERATION ASSAY

Cells were seeded (2×10^3cells/well) in triplicates in a 96-well plate, allowed to adhere for 24 h at 37°C and cultured for further 24, 48, 72, and 96 h, respectively. After each time period, media was removed and 50 μl of MTT (methylthiazole tetrazolium, 1 mg/ml in DMEM without phenol red) was added in each well and incubated for 4 h at 37°C. Formazan crystals thus formed were solubilized in 50 μl of iso-propanol by incubating with shaking for 10 min at room temperature. Absorbance was measured at 570 nm using 630 nm as reference filter.

TRITIATED THYMIDINE ACCUMULATION ASSAY

MCF-NG and MCF-HG cells (5×10^3/well) were plated in a 96-well plate in their respective media and allowed to grow for 60 h followed by the addition of 100 nCi of tritiated thymidine and incubated further for 12–14 h. Cells were trypsinized with 50 μl of TPVG per well and harvested on a glass membrane using Filtermate cell harvester (Packard, Albertville). Incorporation of tritiated thymidine in cellular DNA was quantified by using a Top Count Micro plate Scintillation Counter (Packard, Albertville).

CLONOGENIC SURVIVAL ASSAY

Five hundred cells were plated in 35 mm petri plates and allowed to grow and form colonies for 12 days with media being changed every third day. Colonies formed were fixed with 3% para-formaldehyde and stained with 0.05% crystal violet. Photographs were taken with Versa Doc (Bio-Rad, Hercules, CA).

SENESCENCE ASSOCIATED β-GALACTOSIDASE STAINING

MCF-NG and MCF-HG cells were plated at a density of 250 cells per well in a 48-well plate and allowed to grow for 2 weeks. For senescence-associated β-galactosidase (SA-β-Gal) staining, cells were washed twice with PBS and fixed with 2% para-formaldehyde for 5 min. Cells were washed again with PBS and incubated at 37°C (without CO_2) with fresh 1 mg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) made as 40 mg/ml stock in diethylformamide containing 5 mM potassium ferrocyanide, 150 mM NaCl, 40 mM citric acid/sodium phosphate, pH 6.0, and 2 mM $MgCl_2$. Cells were examined for the development of blue color, which was evident after 12–16 h of incubation with X-Gal. Doxorubicin (1 μM for 2 h) treated MCF-7 cells were taken as a positive control for SA-β-Gal staining done after 2 days of the drug removal. Cells were finally rinsed with PBS (1×) and photomicrographs were taken with DP-71 camera (Olympus, Tokyo, Japan).

MTT CYTOTOXICITY ASSAY

MCF-7 cells were seeded at a density of 7,500 cells per well in 96-well plates and allowed to adhere for 24 h at 37°C. Next day, cells were treated with various concentrations of carboplatin or 5-FU for 36 h. After that the media was removed and 50 μl of MTT (methylthiazole tetrazolium, 1 mg/ml in DMEM without phenol red) was added to each well and incubated for 4 h at 37°C. Formazan crystals thus formed were solubilized in 50 μl of iso-propanol by incubating with shaking for 10 min at room temperature. Absorbance was measured at 570 nm using 630 nm as reference filter.

DRUG ACCUMULATION ASSAY

C14 labeled 5-fluorouracil (^{14}C-5-FU) accumulation was measured in MCF-NG and MCF-HG cells. Cells (1×10^5/well) were grown in 24-well plates and incubated with 500 μCi of ^{14}C-5-FU for 24 h. Thereafter cells were washed thrice with chilled PBS (1×), lysed with

0.1% SDS and radiolabeled drug incorporated in the cells was quantified by using Top Count Micro plate Scintillation Counter (Packard, Albertville).

RNA EXTRACTION, cDNA SYNTHESIS, AND RT-PCR

Total cellular RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad), according to the manufacturer's instructions. Five micrograms of total RNA and oligo (dT) 12–18 primers were taken in diethyl pyrocarbonate treated water. cDNA synthesis was initiated using 200 units of M-MLV reverse transcriptase (Invitrogen, Carlsbad) under conditions recommended by the manufacturer and the reaction was allowed to proceed at 37°C for 90 min. Reaction was terminated by heating at 70°C for 5 min. Each RT-PCR contained 15% of cDNA, 20 pmol of each primer in 20 mM Tris–HCl (pH 8.4) containing 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, and 1 unit of platinum Taq DNA polymerase (Invitrogen, Carlsbad) in a final volume of 25 μl. After an initial denaturation for 30 s at 94°C, 35 and 45 cycles (for β-Actin and MDR-1, respectively) of denaturation (94°C for 1 min), annealing (for 1 min), and extension (72°C for 2 min) were performed on a DNA thermal cycler (Techne, Cambridge, UK) with a final extension for 10 min at 72°C. The primer pairs used were as follows: MDR-1 5′-AAGCTTAGTACCAAAGAG GCTCTG-3′ (F), 5′-GGCTAGAAACAATAGTGAAAACAA-3′ (R) and $\tilde{\beta}$-Actin 5′-ATCTGGCACCACACCTTCTACAATGAGCTGCG-3′ (F), 5′-CGTCATACT CCTGCTTGCTGATCCACATCTGC-3′ (R). The annealing temperature used for MDR-1 and β-Actin was 57°C.

CELLULAR PROTEIN PREPARATION AND IMMUNOBLOTTING

Following treatments, cells were washed thrice with ice-cold PBS and lysed in ice-cold lysis buffer (50 mM Tris–HCl, pH 7.5, with 120 mM NaCl, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM EDTA, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1% NP-40 and protease inhibitor cocktail). Lysates were repeatedly passed through a 27-gauge needle and centrifuged at 12,000 rpm for 30 min. Equal amount of protein samples were resolved on 8–10% SDS-polyacrylamide gel and transferred onto PVDF membrane. The membranes were probed with antibodies against Cyclin E, cdk-2, Phospho-Akt (Ser-473), Akt, PARP, β-Actin (Santa Cruz Biotechnology, CA), and P-gp (Abcam). Whenever required, blots were stripped by incubating membrane at 50°C for 10 min in stripping buffer (62.5 mM Tris–Cl pH 6.7, 100 mM 2-mercaptoethanol, 2% SDS) with intermittent shaking. Membranes were washed thoroughly with TBS and reprobed with required antibodies. Otherwise, gels run in duplicates were probed for desired proteins by western blotting and compiled together. Most of the experiments were repeated and representative blots are shown in figures.

MEASUREMENT OF REACTIVE OXYGEN SPECIES BY FACS

Approx 5×10^5 cells were plated in 35 mm petri plates and allowed to adhere for 24 h. Cells were pre-treated with 10 mM NAC for 4 h followed by treatment with desired concentrations of carboplatin and 5-FU or alone for 36 h and control cells were vehicle treated. Cells were then washed with PBS (1×, 4°C) and collected in 1 ml respective media after trypsinization and centrifuged at 2,500 rpm for 5 min. Media was discarded and 10 μM CM-H2DCFDA (chloromethyl-2′,7′-dichlorofluorescein diacetate) in 1 ml cold PBS was added per sample. Cells were then incubated in dark at room temperature for 25 min and centrifuged at 2,500 rpm at 4°C. Cells were again washed twice with chilled PBS (1×). They were resuspended in 500 μl chilled PBS (1×) and fluorescence intensity of CM-H2DCFDA was recorded by FACS Calibur (Becton Dickinson, CA). Data were analyzed using cell quest software for 10,000 cells.

CYTONUCLEAR FRACTIONATION FOR IMMUNOBLOTTING

Cells were plated in 60 mm petri plates and after desired treatment, washed with chilled 1× PBS once. Cells were collected by scraping and transferred to eppendorf tubes, centrifuged at 250*g* for 5 min at 4°C and washed twice with chilled 1× PBS. These were resuspended in chilled 1× cytoplasmic lysis buffer (Chemicon, Billerica) containing 0.5 mM DTT and protease inhibitors, incubated on ice for 15 min, centrifuged at 250*g* for 5 min, and supernatant was discarded. The pellet was resuspended in chilled cytoplasmic lysis buffer (1×). Cell suspension was then passed through 27-gauge syringe needle five times. Disrupted cell suspension was centrifuged at 8,000*g* for 20 min at 4°C. Supernatant was collected as cytosolic fraction and stored at −80°C. Pellet obtained was resuspended in ice cold 1× nuclear extraction buffer (Chemicon, Billerica) containing 0.5 mM DTT and protease inhibitor cocktail. This suspension was sonicated for 15 s at 30% amplitude and then passed through 27-gauge needle five times. Lysate was centrifuged at 16,000*g* for 5 min at 4°C. Supernatant was collected as nuclear fraction and stored at −80°C. Protein content in these fractions was estimated and equal amount of protein was loaded on 10% gel and used for immunoblotting of p65.

LUCIFERASE ASSAY

All cell transfection experiments were carried out by lipofectamine 2000 reagent (Invitrogen, Carlsbad) according to manufacturer's protocol. Semi-confluent cells were transfected with 6 μg of DNA. Each transfection mixture contained 5 μg of pNF-κB-Luc [Singh and Bhat, 2004] and 1 μg of EGFP expression vector pEGFPN1 (BD Biosciences, CA) as an internal control for transfection efficiency. Eighteen hours after transfection, cells were washed and fresh medium was added. Subsequently, cells were treated with 250 μM of carboplatin or 450 μM 5-FU for 36 h. The transfection efficiency was monitored by cotransfection of GFP-expressing vector pEGFPN1. Cells were harvested, and luciferase assays were performed using an assay kit (Promega, Madison). The luciferase readings were taken in luminometer (Fluoroskan Ascent FL, Labsystems, Finland) and further normalized with GFP to calculate relative fold activation. All transfections were performed in triplicates.

STATISTICS

Data are expressed as the mean and standard deviation. Statistical comparisons were made using Student's two-tailed unpaired *t* test and *P* value <0.05 was considered significant.

RESULTS

HYPERGLYCEMIA INCREASES PROLIFERATION OF BREAST CANCER CELLS BY INCREASING EXPRESSION OF CELL CYCLE REGULATORY PROTEINS

Hyperglycemia has been shown to enhance proliferation of various cells including cancer cells [Yamamoto et al., 1999; Okumura et al., 2002]. We cultured MCF7 cells chronically in normoglycemic (MCF-NG) and hyperglycemic (MCF-HG) conditions. MCF-HG cells exhibited an increased proliferative phenotype as compared to MCF-NG and MCF-MNTL cells. The doubling time of MCF-HG cells was found to be 32 h as compared to 46 h for MCF-NG cells (Fig. 1A). This observation was further supported by increased incorporation of tritiated thymidine in MCF-HG cells (inset, Fig. 1A). Also, the number of colonies formed by MCF-HG cells was more as detected by crystal violet staining (Fig. 1B). In our quest to probe for the cell cycle regulatory molecules involved, we detected increased expression of cyclin E and cdk-2 in cells cultured under HG condition (Fig. 1C). Chronic exposure to elevated glucose level did not have effect on senescent properties as detected by staining the cells for SA β-Gal (Fig. 1D). As a control for osmolarity MCF-7 cells in NG medium were supplemented with 19.5 mM mannitol chronically (MCF-MNTL) and no significant change in growth properties was detected in these cells in comparison with MCF-NG cells (Fig. 1A and C).

HYPERGLYCEMIA ENHANCES CYTOTOXICITY AND REDUCES IC50 VALUES FOR CARBOPLATIN AND 5-FU IN MCF-7 AND HepG2 CELLS

To investigate whether hyperglycemia affects kinetics of cell death induced by carboplatin and 5-FU, MCF-NG, and MCF-HG cells were treated with increasing concentration of these drugs for 36 h. IC50 values were calculated by measuring cell survival using MTT cell proliferation assay. Fifty percent cell death in MCF-NG cells was observed with 380 μM carboplatin and 580 μM 5-FU, whereas IC50 values for MCF-HG cells were 250 and 450 μM for carboplatin and 5-FU, respectively (Fig. 2Aa and 2Ab). We then treated MCF-NG and MCF-HG cells with 250 μM carboplatin and 450 μM 5-FU for 24 and 48 h. It was observed that in MCF-HG cells, death was more at 24 and 48 h treatment points though in either conditions both drugs induced cytotoxicity in a time dependent manner. No changes in cell death were observed in drug treated MCF-MNTL cells in comparison with MCF-NG cells (Fig. 2B, a and b). The differential sensitivity of these drugs is also evident by increased PARP cleavage in MCF-HG cells in comparison to the MCF-NG cells treated with respective drug (Fig. 2C). Increased cell death in hyperglycemic condition was also observed in hepatocellular carcinoma HepG2 cells (unpublished data) indicating that hyperglycemia may affect therapeutic potential of DNA damaging drugs in various cancer types.

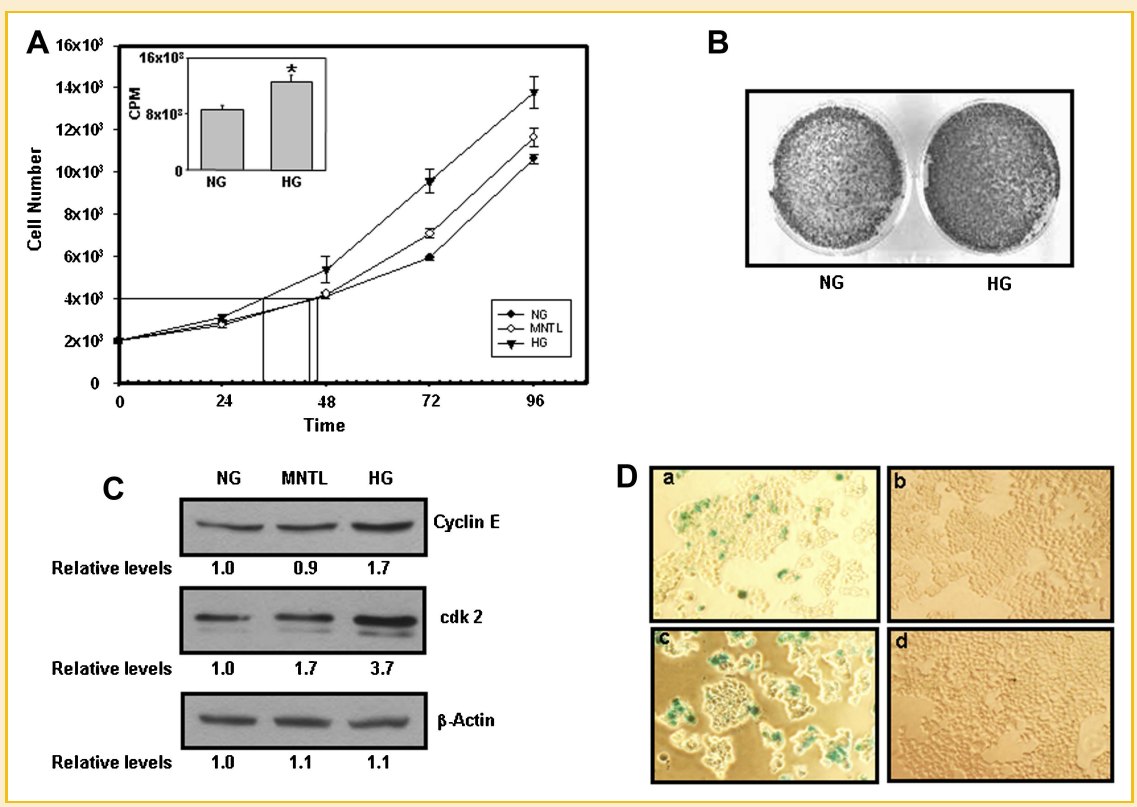

Fig. 1. Hyperglycemia increases proliferation of breast cancer cells by increasing expression of cell cycle regulatory proteins. A: Growth kinetics of MCF-NG and HG cells by MTT assay, (inset, tritiated thymidine accumulation assay). B: Colony formation assay. C: Expression levels of cell cycle regulatory proteins cyclin E and cdk-2. D: Senescence associated β-galactosidase activity (b) MCF-NG and (d) MCF-HG cells (a) and (c) blue stained doxorubicin treated MCF-NG and MCF-HG cells, respectively, served as positive control. $^{*}P < 0.05$.

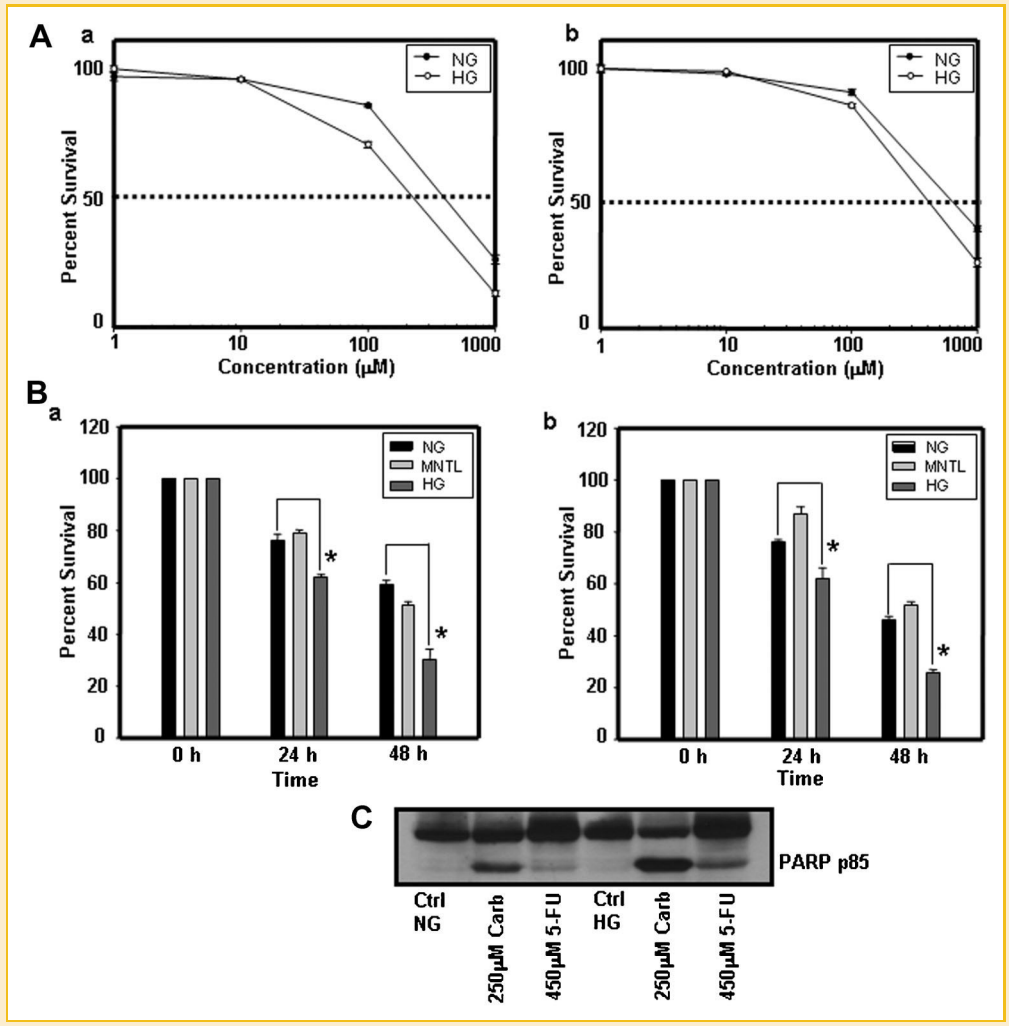

Fig. 2. Hyperglycemia enhances cytotoxicity of carboplatin and 5-FU. A: Dose dependent inhibition of growth in MCF-NG and MCF-HG cells by (a) Carboplatin, (b) 5-FU. B: Time dependent inhibition of growth in MCF-NG, MCF-HG, and MCF-MNTL cells by (a) Carboplatin (250 μM), (b) 5-FU (450 μM). C: Relative PARP cleavage in MCF-NG and MCF-HG cells upon treatment with 250 μM carboplatin and 450 μM 5-FU. $^{*}P < 0.05$.

HYPERGLYCEMIA INCREASES DRUG ACCUMULATION DUE TO REDUCED P-gp LEVELS

Based on our observation that hyperglycemia enhances the cytotoxicity of carboplatin and 5-FU, we investigated whether this is a consequence of altered drug accumulation. Accumulation of C-14 labeled 5-FU for 24 h increased significantly by 1.9-fold in MCF-HG cells (Fig. 3A). However, in HepG2 cells we did not observe any changes in drug accumulation in response to increased glucose levels (unpublished data). We then checked MDR-1 mRNA and P-gp protein levels in MCF-NG and MCF-HG cells. Interestingly, MDR-1 mRNA and P-gp protein levels in MCF-HG cells were reduced in comparison to MCF-NG cells (Fig. 3B). To ascertain that elevated P-gp protein levels in MCF-NG cells is associated with diminished drug retention and reduced cytotoxic effect, we used verapamil (VPL) to specifically inhibit P-gp [Woodland et al., 2003]. MCF-NG cells were pretreated with 40 μM VPL and ^{14}C-5FU accumulation increased by ~1.4-fold in MCF-NG cells (Fig. 3C). To confirm that the increase in drug accumulation upon treatment with VPL is consequence of decrease in P-gp, we checked the expression level of P-gp in NG and HG cells. On treatment with 40 μM VPL, P-gp levels decreased by 1.9- and 1.5-fold in MCF-NG and MCF-HG cells, respectively (Fig. 3D). Also, treatment with VPL increases cytotoxic activity of carboplatin and 5-FU in MCF-NG cells. Under identical experimental conditions VPL treatment did not significantly change drug induced killing of MCF-HG cells (Fig. 4A and B). VPL (40 μM) treatment reduces the viability of both MCF-NG and HG cells. It appears VPL effects NG cells more which may be associated with the levels of P-gp protein.

HYPERGLYCEMIA INDUCED INCREASE IN CYTOTOXICITY IS MEDIATED VIA ROS DEPENDENT MECHANISMS

Hyperglycemia increased ROS levels by approximately 1.4-fold in MCF-HG cells compared to MCF-NG cells. Furthermore, ROS levels in carboplatin and 5-FU treated MCF-HG cells increased by 1.6- and

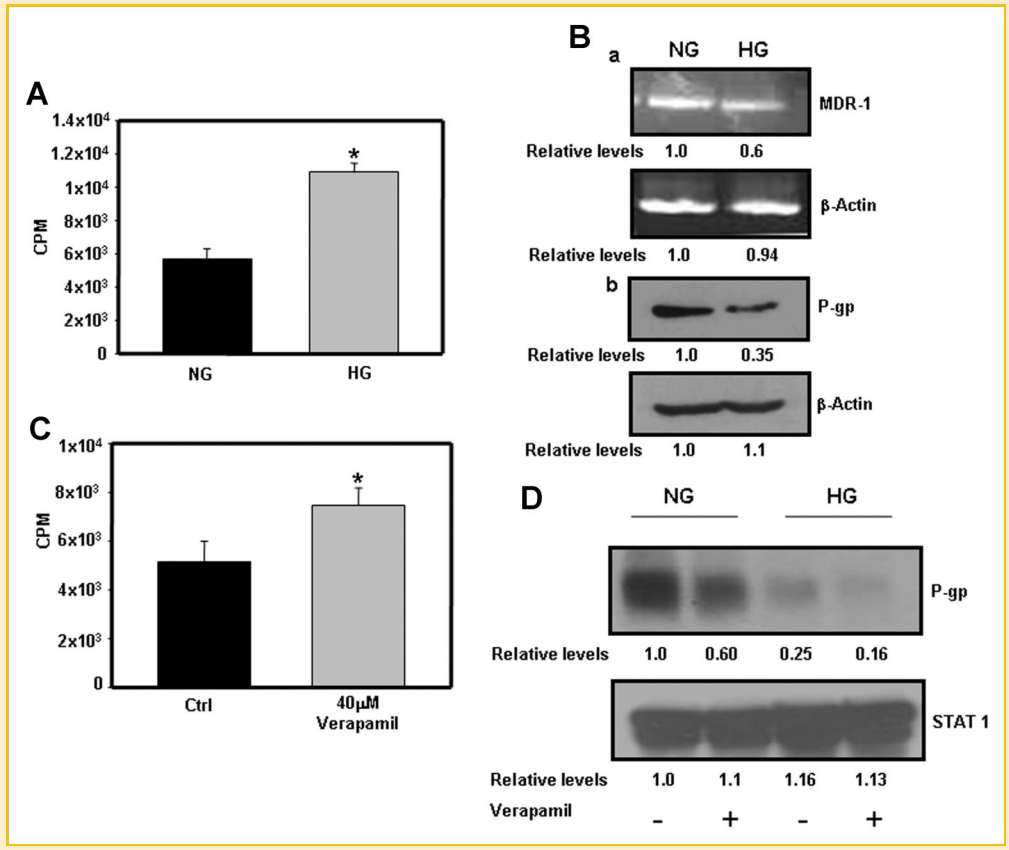

Fig. 3. Hyperglycemia increases drug accumulation and reduces P-gp levels. A: Radiolabeled drug accumulation in MCF-NG and MCF-HG cells. B: Relative expression of (a) MDR-1 at transcriptional level by RT-PCR, (b) P-gp by immunoblotting in MCF-NG and MCF-HG cells. C: Drug accumulation in MCF-NG cells by inhibiting P-gp. Upon treatment with 40 μM verapamil, accumulation of ^{14}C 5-FU is increased in MCF-NG cells. D: P-gp by immunoblotting in MCF-NG and MCF-HG cells in presence or absence of 40 μM Verapamil. $^{*}P < 0.05$.

1.7-fold above the basal level (Fig. 5). Similar increase in ROS levels was detected in drug treated MCF-NG cells (Fig. 5). We pre-treated MCF-HG cells with 100 μM and 1 mM *N*-Acetylcysteine (NAC), a known inhibitor of ROS followed by treatment with drug. Interestingly, survival increased by 30 and 18% in NAC pre-treated MCF-HG cells exposed to carboplatin and 5-FU, respectively (Fig. 6A). This is also evident by the reduction in cleavage of PARP to p85 in cells pre-treated with 1 mM NAC (Fig. 6B). We then measured ROS levels in MCF-NG and MCF-HG cells treated with drug alone and with NAC and drug together. A significant decrease in ROS level was detected, which was more in HG cells as compared to NG cells. The decrease in ROS levels upon carboplatin treatment in the presence of NAC with respect to the carboplatin alone treated cells was 1.17-fold in MCF-NG cells and 1.37-fold in MCF-HG cells (Fig. 6C). Similarly, decrease in ROS levels upon 5-FU treatment in the presence of NAC with respect to the 5-FU alone treated cells was 1.15-fold in MCF-NG cells and 1.47-fold in MCF-HG cells (Fig. 6C).

ENHANCED CELL DEATH IS CAUSED BY ALTERATION IN SURVIVAL MOLECULES

Both carboplatin and 5-FU induce apoptosis (Figs. 2C and 6B) and to explore the molecular basis of enhanced cytotoxicity of these drugs in MCF-HG cells we probed for the activation status of Akt, a known survival factor [Marte and Downward, 1997]. In MCF-HG cells, basal pAkt level is low which is reduced further by carboplatin and 5-FU treatment (Fig. 7A). Akt exerts a direct positive effect on the activation of transcription factor NF-κB, which facilitates transcription of many genes involved in various aspects of cell survival and carcinogenesis [Karim and Lin, 2002; Karin et al., 2002]. Drug treatment causes reduction in the nuclear levels of p65 in NG and HG cells and also diminishes transcriptional activity of NF-κB (Fig. 7B and C). These results support the existing literature that death induced by carboplatin and 5-FU in MCF-NG and HG cells involves reduced pAkt levels and decreased nuclear localization as well as transcriptional activation of NF-κB, thereby causing PARP cleavage and apoptosis.

DISCUSSION

A large pool of data as an evidence for direct and indirect relevance of diabetes on the occurrence, progression, and pathogenesis of breast cancer has been accumulated [Wolf et al., 2006; Larsson et al., 2007; Lipscombe et al., 2008]. Relevance of diabetes in the

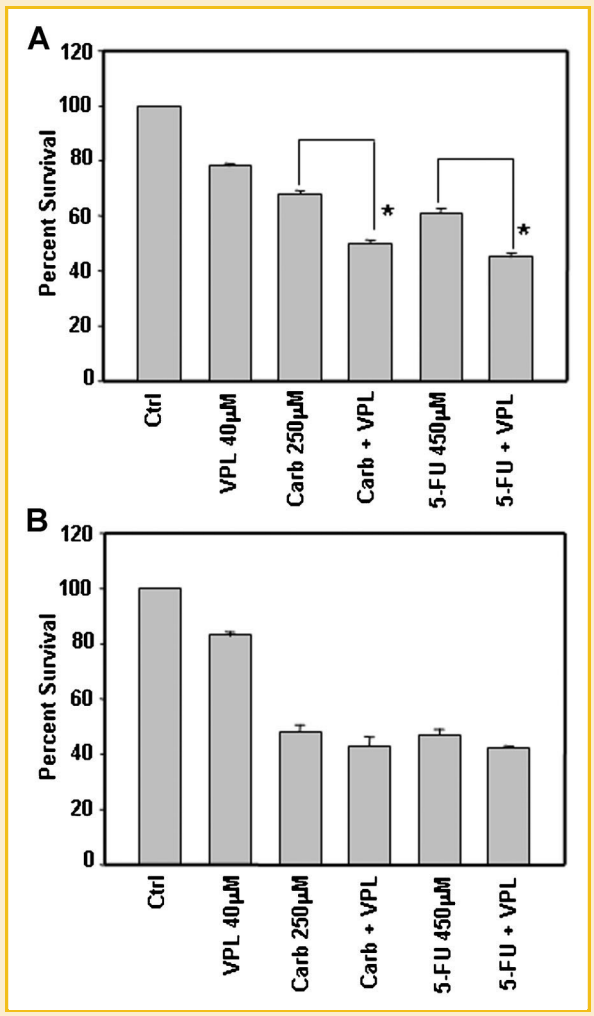

Fig. 4. Verapamil treatment enhances the cytotoxicity of carboplatin and 5-FU. MTT assay (A) MCF-NG and (B) MCF-HG cells in the presence of verapamil (VPL, 40 μM). Cells were pre-treated for 2 h with 40 μM VPL and then treated with 250 μM carboplatin and 450 μM 5-FU in the presence of VPL for 36 h. Cell viability was then estimated by MTT assay as described in material and methods section. VPL selectively enhances the cytotoxicity of carboplatin and 5-FU in MCF-NG cells. $^{*}P < 0.05$.

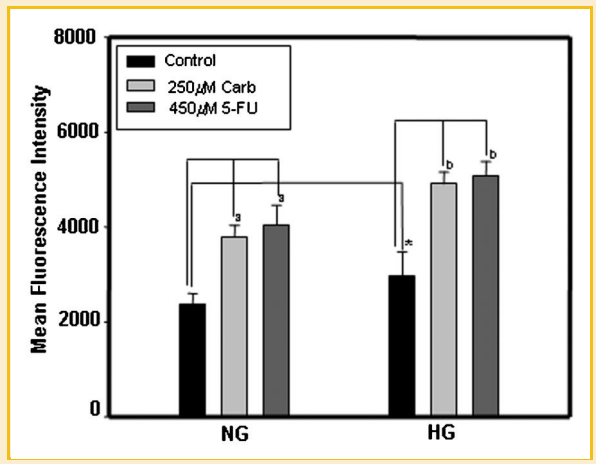

Fig. 5. ROS levels (basal and drug treated) in MCF-NG and MCF-HG cells. Cells were treated with 250 μM carboplatin and 450 μM 5-FU for 36 h. Cells were then harvested by trypsinization, labeled with 10 μM CM-H2DCFDA and fluorescence intensity was measured as described in Material and Methods section. Hyperglycemia increases the basal level of ROS in MCF-HG cells and treatment with carboplatin and 5-FU further elevates the ROS levels.

pathogenesis and clinical course of tumors in breast cancer has been controversially debated [Muti, 2004]. Also, the frequent use of carboplatin or 5-FU and increased prevalence of diabetes in general population serves as an incentive for studies investigating complications associated with cancer chemotherapy particularly in breast cancers.

We mimicked hyperglycemia in vitro and MCF-7 cells exposed chronically to hyperglycemic conditions exhibit enhanced proliferative phenotype because of increased expression of cyclin E and cdk-2. By utilizing this cellular model we investigated relatively unexplored interface of the effect of hyperglycemia on cancer chemotherapy. Our observation that hyperglycemia decreases the IC50 value of carboplatin and 5-FU falls in line with reports which showed increased efficacy of certain anticancer drugs in high glucose conditions [Kung et al., 1963; Sadoff, 1998; Turturro et al., 2007b]. Interestingly, present study for the first time demonstrates that high glucose reduces P-gp level in MCF-7 cells that is preceded by reduction in P-gp mRNA implying regulation of P-gp by hyperglycemia. P-gp, a product of multidrug resistance MDR-1 gene, is an efflux transporter with broad substrate specificity. It binds to a large variety of exogenous and endogenous toxic compounds and exports them to extracellular environment [Stein, 1997; Ambudkar et al., 1999; Gatlik-Landwojtowicz et al., 2004]. Also, increased ROS levels have been shown to decrease P-gp protein in multicellular prostate tumor spheroids [Wartenberg et al., 2001]. Earlier, it has been shown that glucose depletion causes resistance to doxorubicin, a P-gp substrate [Shen et al., 1987]. In addition high glucose is known to reduce the physical barrier properties and membrane fluidity of monolayer cells maintained in 5.5 mM glucose [D'Souza et al., 2003]. Moreover, metabolic state of the cell has been correlated to the modifications in P-gp expression in rodent as well as in human cells which influences MDR-1 status [Sukhai and Piquette-Miller, 2000]. Reduced survival of normoglycemic and hyperglycemic cells in the presence of VPL suggests that it may also affect certain unidentified survival molecules besides decreasing P-gp levels.

Oxidative stress in cancer cells stimulates proliferation, promotes mutations and genetic instability, in addition to causing alteration in cellular sensitivity towards anticancer agents. Hyperglycemia enhances basal ROS level which is further increased by carboplatin and 5-FU in MCF-HG cells. Thus, cumulative increase in ROS above threshold level is likely to be incompatible with cell survival and therefore ROS may exert cytotoxic effect [William et al., 2009]. It has been reported that UV radiation [Uchiumi et al., 1993], low external pH, and osmotic shock [Wei and Roepe, 1994] as well as heat stress [Miyazaki et al., 1992] induced alteration in the

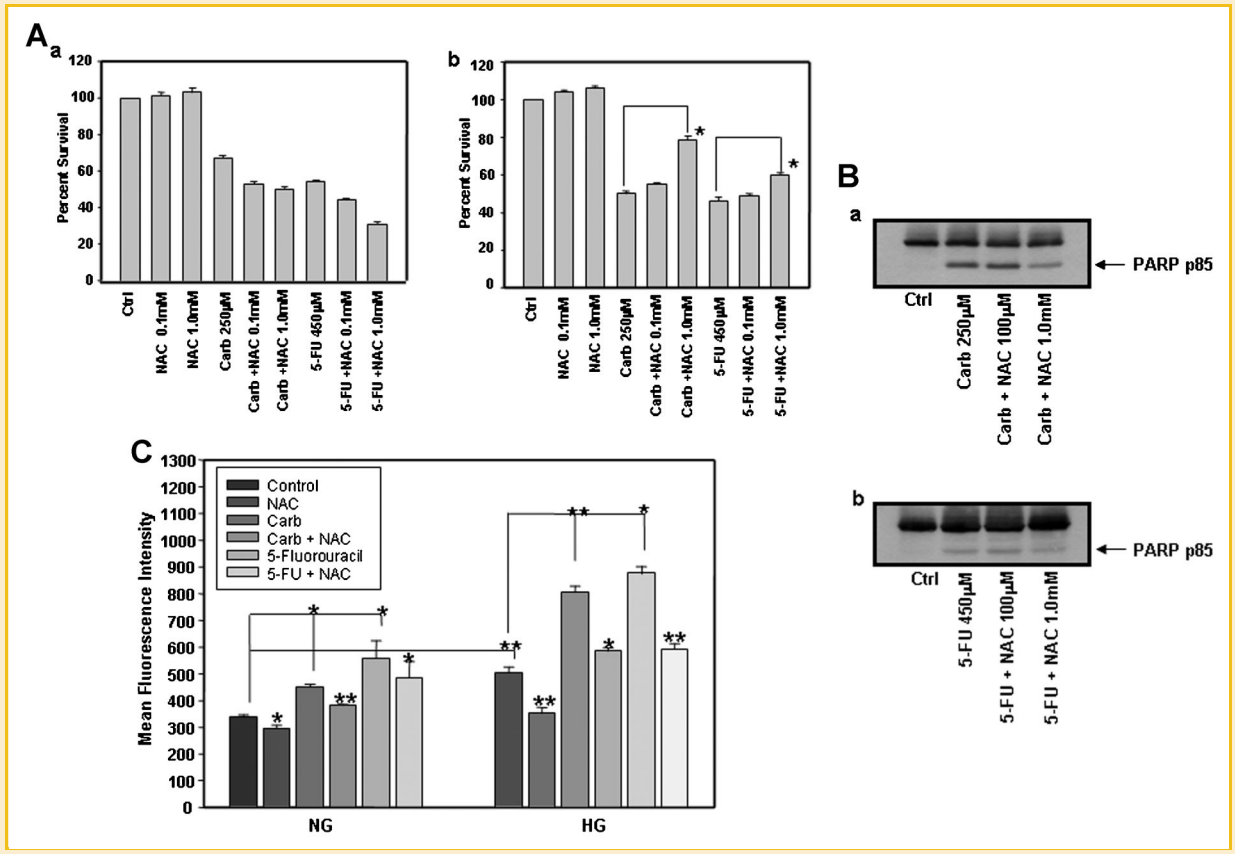

Fig. 6. Treatment with ROS inhibitor NAC causes increased survival in drug treated MCF-HG cells. A: Hyperglycemia induced increase in cytotoxicity is mediated by ROS dependent mechanisms. Cytotoxicity of carboplatin and 5-FU in (a) MCF-NG and (b) MCF-HG cells in the presence of ROS inhibitor NAC. B: PARP cleavage in drug treated (a) MCF-HG and (b) MCF-NG cells in presence of NAC. C: ROS levels in MCF-NG and HG cells in the presence of NAC and NAC +/− carboplatin and 5-FU. Cells were treated with 250 μM carboplatin and 450 μM 5-FU for 36 h alone or with 10 mM NAC pre-treated NG and HG cells. Cells were then harvested by trypsinization, labeled with 10 μM CM-H2DCFDA and fluorescence intensity was measured as described in Material and Methods section. $^{*}P < 0.05$, $^{**}P < 0.01$.

expression of P-gp are accompanied by generation of intracellular ROS which induces apoptosis [Ozben, 2007]. ROS derived from mitochondria is also involved in the initiation phase of apoptosis and cell death signaling. Also, exposure of cancer cells to ROS generating anticancer agents exhausts cellular antioxidant capacity and ROS levels beyond a threshold leads to apoptosis [Ozben, 2007]. Because both hyperglycemia as well as chemotherapeutic drugs induce ROS, it is likely that together this will have profound effect on cell survival. Our results clearly show that NAC scavenges ROS more efficiently in MCF-HG cells. Differential susceptibility of NG and HG cells to the protective effect of NAC may have arisen due to differences in the metabolism and because of intrinsic antioxidants levels [Wang et al., 2010]. Since HG cells are already under increased endogenous oxidative stress a further increase in ROS levels easily predisposes these cells to cytotoxic effect of drugs because the antioxidant tolerance capacity of these cells may be operating at its maximum. On the contrary, this may not be the case with NG cells and thus an exogenous scavenger NAC shows the effect selectively in HG cells whose intracellular antioxidant capacity may be already exhausted. Following 1 mM NAC treatment, increased death observed in carboplatin treated MCF-HG cells was almost completely reversed, whereas partial increase in cell survival was observed in 5-FU treated cells. These results indicate that increased ROS generation under hyperglycemic conditions completely or partially is responsible for enhanced cell death depending on the drug used.

Survival kinases as well as growth promoting transcriptional factors are constitutively activated in various cancer cells. Akt, a serine/threonine kinase governs cell survival and oncogenesis directly by phosphorylation and inactivation of several pro-apoptotic targets [Brunet et al., 1996; Marte and Downward, 1997]. Depending upon the drugs used, treatment reduces activation of Akt in both MCF-NG and HG cells. Also, basal activation of Akt was less in untreated HG cells when compared to NG cells. These findings are in agreement with the reports in other cells in which hyperglycemia is known to reduce pAkt levels [Song et al., 2007; Popov et al., 2009]. In addition, it has been previously shown that persistent nuclear localization and constitutive activation of NF-κB is important for survival and oncogenesis in tumors of breast, ovarian, colon, thyroid, prostate, and melanoma [Rayet and Gelinas, 1999; Darnell, 2002]. Studies from our laboratory and those of others have documented that carboplatin and 5-FU induce apoptosis

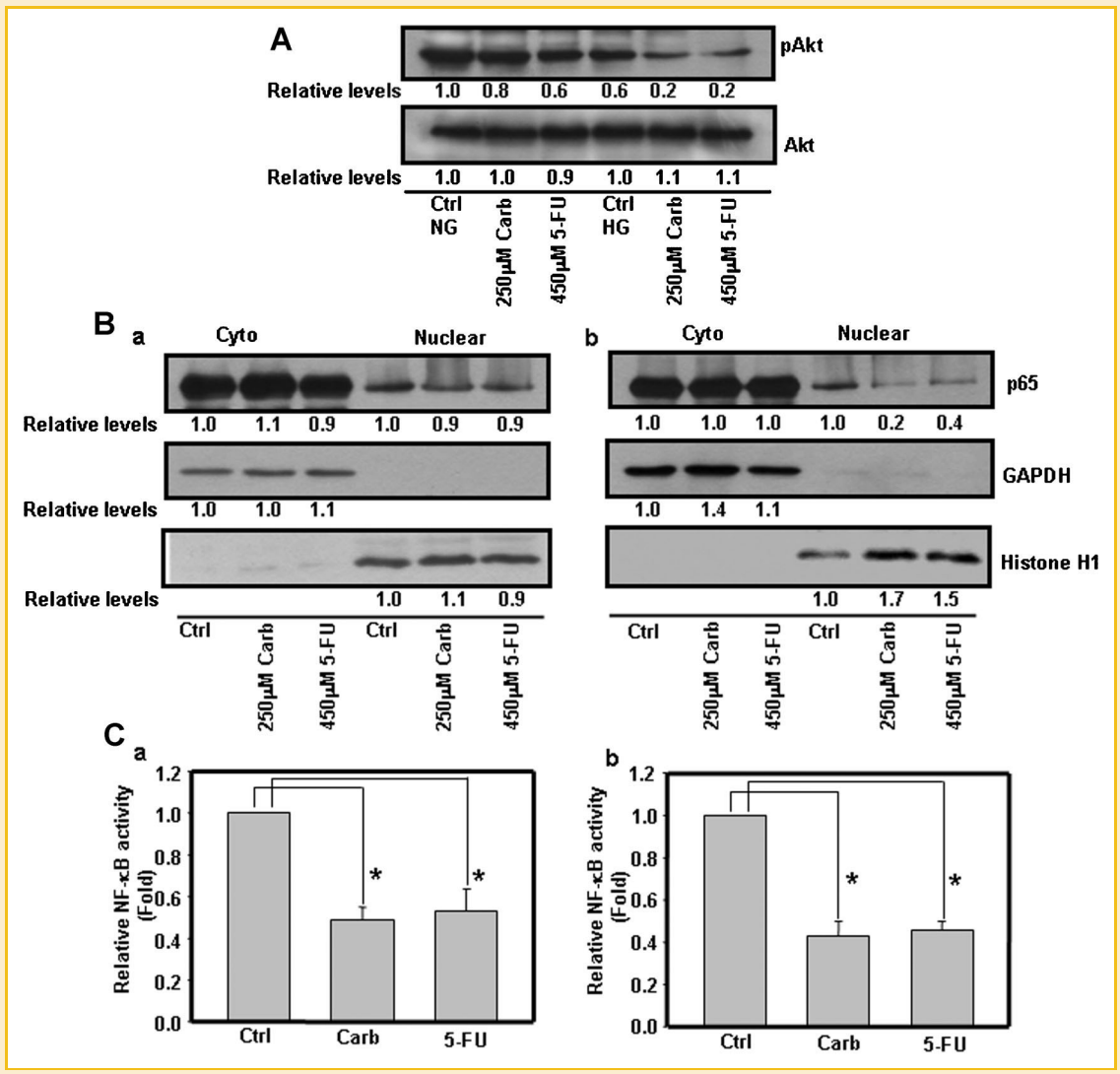

Fig. 7. Cytotoxicity of carboplatin and 5-FU is associated with reduced phosphorylation of Akt and reduced transcriptional activity of NF-κB. A: Expression levels of active phosphorylated form of Akt in MCF-NG and MCF-HG cells before and after treatment with carboplatin and 5-FU. B: Reduced nuclear localization of p65 subunit of NF-κB in (a) MCF-NG and (b) MCF-HG cells upon treatment with carboplatin and 5-FU. C: Treatment with carboplatin and 5-FU downregulates the transcriptional activity of NF-κB in (a) MCF-NG and (b) MCF-HG cells. $^{*}P < 0.05$.

in diverse cell types by down-regulating NF-κB regulated pathways [Heckman et al., 2002; Singh and Bhat, 2004]. Thus, glucose levels do not seem to have effect on cell death mechanism initiated by carboplatin and 5-FU per se.

CONCLUSIONS

Collectively, our study supports the idea that hyperglycemia renders MCF-7 breast cancer cells more susceptible to oxidative stress. We propose that metabolic status induced oxidative stress along with DNA damage has a potential to maximize the toxicity of DNA damaging drugs. We conclude that hyperglycemia favors cytotoxicity induced by carboplatin and 5-FU and decreases their IC50 in MCF-7 cells. Increased cytotoxicity of these drugs is accomplished because of synergistic effect of hyperglycemia-mediated reduction in P-gp levels as well as increased drug accumulation which enhances ROS levels in elevated glucose conditions. It is likely that enhanced proliferation of MCF-7 cells under hyperglycemic conditions may also predispose cells to cytotoxic agents because anticancer drugs are more effective in rapidly proliferating cancer cells (Fig. 8). Collectively, our results provide evidence and mechanistic insight into impact of hyperglycemia on cancer chemotherapy. Present study could have clinical implications because the major metabolic alteration in diabetic patients is of elevated glucose and thus supply of glucose to the cells along with the nutritional state of the patient may influence efficacy and toxicity of chemotherapeutic agents. These findings however do not

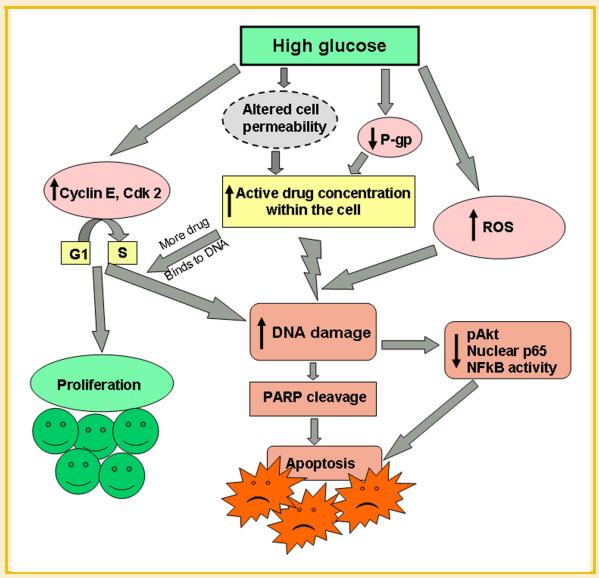

Fig. 8. An overview of the mechanisms which contribute towards increased cytotoxicity of DNA damaging drugs in the presence of high glucose. Hyperglycemia induced changes in metabolic status of cancer cells which promote oxidative stress and DNA damage have the potential to maximize the toxicity of DNA damaging drugs. Increased cytotoxicity is accomplished because of synergistic effect of hyperglycemia-mediated reduction in P-gp expression and altered cellular permeability (leading to increased accumulation of drugs) and enhanced ROS levels. Additionally, it is likely that enhanced proliferation of cancer cells under hyperglycemic conditions may also play a role in predisposing these cells to cytotoxic efficacy because anticancer drugs are more effective in rapidly proliferating cells.

rule out the possibility that high glucose may also predispose patients to increased generalized toxicity to chemotherapy which needs to be scientifically and clinically verified and validated.

ACKNOWLEDGMENTS

The authors thank Dr. G.C. Mishra, Director, NCCS for being very supportive and giving all the encouragement to carry out this work. They also thank Department of Biotechnology, Government of India for providing financial support. VP and BC thanks Council for Scientific and Industrial Research (CSIR), New Delhi, for providing fellowship. They also thank FACS facility at NCCS for assistance. This work was done in partial fulfillment of a Ph.D. thesis (of VP) submitted to the University of Pune, India.

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