High glucose and insulin differentially modulates proliferation in MCF-7 and MDA-MB-231 cells

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Abstract

Various preclinical and clinical studies have linked diabetes and breast cancer, but little is known regarding the molecular mechanism involved. This study aimed to investigate the effect of high glucose and insulin in breast cancer cells (MCF-7: non-invasive, hormone dependent, and MDA-MB-231: invasive, hormone independent). In contrast to MCF-7 cells, high glucose augmented proliferation of MDA-MB-231 cells as observed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and bromodeoxy-uridine assays. The high-glucose condition led to increased expression of cyclin D1, de-phosphorylation of p38, and increased phosphorylation of ERK in MDA-MB-231 cells but not in MCF-7 cells. Interestingly, we observed increased phosphorylation of GSK-3 β , NF- κ B, and ER α only in MCF-7 cells, highlighting their role as potential targets in prevention of progression of breast cancer under a high-glucose and insulin condition. Furthermore, insulin treatment under a high-glucose condition resulted in increased histone H3 phosphorylation and de-acetylation only in MDA-MB-231 cells. Taken together, we provide the first evidence that high glucose and insulin promotes proliferation of MDA-MB-231 cells by differential alteration of GSK-3 β , NF- κ B, and ER α expression and histone H3 modifications, which may directly or indirectly modulate the expression of genes involved in its proliferation.

Key Words

- high glucose
- insulin
- cell proliferation
- histone H3 modifications

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Introduction

Breast cancer is currently the most common type of cancer, affecting one of every eight women in the USA (Wolf *et al.* 2006). The major risk factors for breast cancer are genetic predisposition, obesity, and old age, which also significantly contribute to diabetes mellitus. Diabetes is a metabolic syndrome characterized by hyperglycemia, hyperinsulinemia, and insulin resistance. It has been recognized as a risk factor for various malignancies, including breast, endometrial, colon, and pancreatic cancers (Czyzyk & Szczepanik 2000). It has been reported that up to 16% of older breast cancer patients also suffer from diabetes (Coebergh *et al.* 1999). Many epidemiological studies suggest that women

with diabetes have increased risk of breast cancer (Xue & Michels 2007, Suh & Kim 2011, Boyle *et al.* 2012). A recently conducted meta-analysis provides evidence that diabetes is associated with 23% increased risk of breast cancer, especially in post-menopausal women (Liao *et al.* 2011). Diabetes induces a variety of changes in the hormonal systems including insulin, insulin-like growth factor, estrogen, and other growth factors, which may interact in a complex and intricate manner, thus playing an important role in the promotion of breast carcinogenesis (Lai *et al.* 2001).

As cancer cells use glucose as the source of energy for their proliferation, hyperglycemia provides a favorable

environment for the growth and survival of breast cancer cells (Beckner *et al.* 1990). Hyperglycemia has been reported to promote pancreatic cancer in BxPC-3 and MIA PaCa-2 cells in a concentration-dependent manner (Liu *et al.* 2011a). It has been reported that cancer-specific survival of patients with hyperglycemia was much less than that of patients with normoglycemic levels (Villarreal-Garza *et al.* 2012). Further, it has been reported that hyperglycemia confers resistance to chemotherapy in malignant breast cancer cells but not in non-malignant cells (Zeng *et al.* 2010). Insulin is known to have mitogenic action in normal mammary tissue as well as in breast cancer cells (Papa & Belfiore 1996). Hyperinsulinemia with insulin resistance is a significant risk factor for the development of breast cancer (Papa *et al.* 1997). Ferguson *et al.* (2012) have recently shown that high systemic insulin levels promote breast cancer metastasis in a hyperinsulinemic mouse model by activating *c-myc* signaling. It has been shown that high-dose human insulin and its analogs promoted T24 (bladder cancer cell), HCT-116 (colorectal cancer cell), and PC-3 (prostate cancer cell) proliferation (Weinstein *et al.* 2009, Liu *et al.* 2011b). These reports indicate that metabolic changes occurring in patients with hyperglycemia and hyperinsulinemia lead to increased susceptibility of breast cancer progression. This study was designed to investigate the effect of high glucose and insulin on proliferation in MCF-7 (hormone dependent) and MDA-MB-231 (hormone independent) breast cancer cells.

Materials and methods

Chemicals

DMEM, fetal bovine serum (FBS), and insulin were purchased from Sigma Chemical Co. Antibiotic solution (penicillin and streptomycin) and trypsin-EDTA solution were purchased from Gibco. 5,6-Chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-DCF-DA) was purchased from Molecular Probes (Invitrogen). ECL detection kit and ECL hyperfilm were obtained from Amersham Bioscience. Bromodeoxyuridine (BrdU) cell proliferation kit was purchased from Calbiochem (EMD Chemicals, Darmstadt, Germany). All the other chemicals were purchased from Sigma, unless otherwise mentioned.

Cell culture and treatment

MCF-7 and MDA-MB-231 breast cancer cells (procured from ATCC, Manassas, VA, USA), derived from human breast adenocarcinoma, were cultured in DMEM supplemented with 10% FBS and antibiotics (penicillin, 100 IU/ml and

streptomycin, 100 µg/ml) in 5% CO₂ at 37 °C. Cells were cultured in normal-glucose (5 + 20 mM mannitol for osmotic balance) and high-glucose (25 mM) conditions. For western blotting studies, at 80–90% confluence, cells were subjected to 5-h serum starvation in serum-free DMEM and then stimulated with bovine insulin (25 nM) for 30 min under normal- and high-glucose conditions.

Visualization of intracellular reactive oxygen species by DCF-DA staining

After insulin treatment, cells were incubated with CM-H2-DCF-DA (10 µM) in neutral red-free DMEM in darkness for 10 min. Cells were then washed with ice-cold PBS and intracellular reactive oxygen species (ROS) generation was detected by green fluorescence of DCF-DA using a Nikon inverted fluorescent microscope at an excitation wavelength of 488 nm and emission at 515–540 nm. Quantification of DCF fluorescence was done by NIH Image J Software (Bethesda, MD, USA) and the results were expressed as fold change in fluorescent intensity relative to normal control.

MTT cell viability assay

The MTT cell viability assay is based on the conversion of MTT to violet color formazan crystals by mitochondrial dehydrogenase enzymes. Briefly, cells were counted and seeded in 24-well cell culture plates with cell density of 1×10^5 cells/well. Cells were allowed to attach and were treated with insulin (25 nM) for 24 h in normal- as well as in high-glucose conditions. Then, cells were washed with PBS and incubated with MTT (150 µg/ml) for 4 h at 37 °C. Cells were again washed with chilled PBS and DMSO (400 µl) was added in each well to dissolve the formazan crystals formed. Absorbance was measured at 595 nm using a Flexstation III spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) and the results were expressed as percentage cell viability, assuming the viability of control cells was 100%.

BrdU cell proliferation assay

The BrdU cell proliferation assay is a non-isotopic immunoassay for the quantification of BrdU incorporation into newly synthesized DNA of actively proliferating cells. MCF-7 and MDA-MB-231 cells were seeded into a 96-well cell culture plate at a cell density of 1×10^5 cells/ml. Cells were allowed to attach and were treated with insulin (25 nM) for 24 h in normal- as well as in high-glucose conditions. The cells were then incubated

with BrdU for 24 h and the assay was performed further according to the manufacturer's instructions. The absorbance was measured at a dual wavelength of 450–540 nm using a Flexstation III spectrophotometer (Molecular Devices). The result was calculated as percentage of cell proliferation compared with normal glucose control.

Isolation of total proteins and western blotting

Cells were incubated and sub-cultured under normal- or high-glucose conditions for 2 weeks. After serum starvation of 5 h, they were then incubated with insulin for 30 min. After insulin treatment, cells were washed with ice-cold PBS and then lysed in modified low-salt buffer (LSB; 10 mM Tris-HCl, pH 7.4, 150 mM EDTA, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 µg/ml aprotinin, 1 mM sodium butyrate, and 0.05% NP-40). Proteins were electrophoretically transferred onto nitrocellulose membrane using semi-dry transfer apparatus (Bio-Rad). Immunoblot analysis was performed using anti-phospho-p38 (rabbit, 1:500, Santa Cruz Biotechnology, CA, USA), anti-p38 (rabbit, 1:1000, Santa Cruz Biotechnology), anti-phospho-ERK1/2 (rabbit, 1:500, Santa Cruz Biotechnology), anti-ERK (rabbit, 1:500, Santa Cruz Biotechnology), anti-cyclin D1 (rabbit, 1:500, Santa Cruz Biotechnology), anti-phospho-glycogen synthase kinase 3β (GSK-3β) ser 9 (rabbit, 1:500, Cell Signaling, MA, USA), anti-GSK-3β (rabbit, 1:500, Santa Cruz Biotechnology), anti-phospho-nuclear factor-κB (NF-κB; rabbit, 1:500, Santa Cruz Biotechnology), anti-NF-κB (rabbit, 1:500, Santa Cruz Biotechnology, CA, USA), anti-phospho-estrogen receptor α (ERα) ser 118 (rabbit, 1:500, Santa Cruz Biotechnology), anti-ERα (rabbit, 1:500, Santa Cruz Biotechnology), anti-actin (rabbit, 1:1000, Santa Cruz, Biotechnology), anti-α tubulin (mouse, 1:1000, Active Motif, CA, USA), and HRP-conjugated secondary antibodies (Santa Cruz Biotechnology). The antigen-antibody complex was visualized with an ECL detection kit (Amersham Bioscience). For subsequent antibody treatment, membranes were stripped in stripping buffer and re-probed with another antibody. All the immunoblots were repeated three times and quantified by densitometry scanning with NIH Image J Software.

Extraction of histones

Histone proteins were isolated from cells by an acid-soluble extraction method as described earlier (Tikoo *et al.* 2007). Briefly, nuclear pellet was suspended in LSB (10 mM Tris, 10 mM NaCl, 10 mM EDTA, 10 µg/ml aprotinin,

10 µg/ml leupeptin, and 1 mM PMSF). Histones were extracted in 0.25 M HCl and precipitated by adding 20% trichloroacetic acid. The precipitate was washed with acetone-HCl (0.25%) followed by acetone. Immunoblot analysis was performed using anti-phospho-histone H3 ser 10 (rabbit, 1:2500, Santa Cruz Biotechnology), anti-acetylated histone H3 (rabbit, 1:2500, Santa Cruz Biotechnology), anti-histone H3 (goat, 1:2500, Santa Cruz Biotechnology), and HRP-conjugated secondary antibodies (Santa Cruz Biotechnology). Histones were detected and quantified as described previously.

Statistical analysis

All values are expressed as mean $\pm$ S.E.M. Results shown are representative of three different experiments. Statistical comparison between more than two different groups was performed using one-way ANOVA followed by Tukey's test. *P* value <0.05 was considered to be significant.

Results

High glucose and insulin generates ROS in MCF-7 and MDA-MB-231 cells

Oxidative stress has been widely implicated in the initiation and progression of cancer (Seoane *et al.* 2011, Gupta-Elera *et al.* 2012). A significant increase in DCF green fluorescence was observed in MCF-7 cells when cultured in a high-glucose condition (25 mM) compared with a normal-glucose (5 mM) condition. The dose and treatment time of insulin was selected after studying intracellular ROS generation at various doses (10, 25, 50, and 100 nM) for different time points (15 min, 30 min, 1 h, and 2 h; data not shown). Insulin (25 nM) treatment for 30 min in normal- as well as in high-glucose conditions led to increased generation of intracellular ROS (Fig. 1A). In contrast to MCF-7 cells, MDA-MB-231 cells showed generation of ROS *per se* under normal- as well as high-glucose conditions, which was further enhanced by insulin treatment (Fig. 1B). This suggests that metastatic breast cancer cells are under mild oxidative stress, independent of the conditions. This might be playing a critical role in the development of resistance to cell death in MDA-MB-231 cells.

Effect of high glucose and insulin on cell proliferation in MCF-7 and MDA-MB-231 cells

The MTT assay demonstrated that in MCF-7 cells, a high-glucose condition led to decreased cell viability (Fig. 2A),

Figure 1

Representative DCF fluorescent images with respective phase-contrast images, showing insulin (25 nM)-induced generation of ROS after 30-min treatment under NG and HG conditions in MCF-7 cells (A) and MDA-MB-231 cells (B). All the images are taken at the magnification of 100 $\times$ (bar length 100 μ m). (C and D) Histograms showing the quantification of high glucose- and insulin-induced DCF fluorescence in MCF-7 and MDA-MB-231 cells respectively. Results shown are representative of three different experiments. *** P < 0.001, where a vs NG-C, b vs NG-Ins, and c vs HG-C. NG-C, normal glucose control; NG-Ins, normal glucose + insulin; HG-C, high glucose control; and HG-Ins, high glucose + insulin. Full colour version of this figure available via <http://dx.doi.org/10.1530/JME-13-0062>.

whereas in MDA-MB-231 cells, it led to increased cell viability (Fig. 2B). However, we did not observe any change in cell viability after insulin treatment under normal- as well as high-glucose conditions in both MCF-7 and MDA-MB-231 cells. In addition, a BrdU cell proliferation assay also showed that in MCF-7 cells, high glucose and insulin treatment prevented proliferation of cells (Fig. 2C). On the other hand, in MDA-MB-231 cells, we observed that a high-glucose condition promoted cell proliferation compared with a normal-glucose condition (Fig. 2D). These results suggest that in MCF-7 cells, oxidative stress generated by high glucose results in cell death. But, MDA-MB-231, metastatic breast cancer cells, overcome the process of cell death and initiate proliferation of cells.

Alteration in expression of cyclin D1 by high glucose and insulin in MCF-7 and MDA-MB-231 cells

Cyclin D1 is involved in the regulation of cell cycle progression from G₁ to S phase through the formation of active enzyme complexes with cyclin-dependent kinases Cdk4 and Cdk6 (Alao 2007). It plays an essential role in the development and progression of several cancers including breast, esophagus, bladder, and lung cancer (Lin *et al.* 2000, Musgrove 2006, He *et al.* 2007). Our results

showed that in MCF-7 cells, a high-glucose condition led to decreased expression of cyclin D1 compared with a normal-glucose condition (Fig. 3A, lanes HG-C and HG-Ins). By contrast, in MDA-MB-231 cells, a high-glucose condition led to increased expression of cyclin D1 (Fig. 3B, lanes HG-C and HG-Ins). We failed to observe that significant change in cyclin D1 expression due to insulin treatment under normal- as well as high-glucose conditions may be because the duration of insulin treatment was 30 min. only. These results further confirm that high glucose prevents proliferation of MCF-7 cells and promotes proliferation of MDA-MB-231 cells.

Hyperglycemia- and insulin-induced changes in phosphorylation of p38 and ERK in breast cancer cells

ROS are known to mediate several biological responses like proliferation, extracellular matrix deposition, and apoptosis (Martindale & Holbrook 2002). To gain insight into the intracellular signaling stimulated by high glucose- and insulin-induced ROS, we investigated the potential role of MAPKs. MAPKs are known to be involved in cell proliferation (Chen *et al.* 2001). The p38 MAPK pathway is activated by cellular stress and it promotes apoptosis in a variety of cell types (Thornton & Rincon 2009). Our results showed that in MCF-7 cells, phosphorylation of p38 increased significantly by insulin (25 nM) treatment for 30 min under a high-glucose condition compared with normal- and high-glucose conditions

Figure 2

MTT assays showing the effects of high glucose and insulin on percentage cell viability compared with normal-glucose conditions in MCF-7 cells (A) and MDA-MB-231 cells (B). BrdU cell proliferation assays showing the effects of high glucose and insulin on percentage cell proliferation compared with a normal-glucose condition in MCF-7 cells (C) and MDA-MB-231 cells (D). Results shown are representative of three different experiments. * P < 0.05, ** P < 0.01, and *** P < 0.001, where a vs NG-C and b vs NG-Ins. NG-C, normal glucose control; NG-Ins, normal glucose + insulin; HG-C, high glucose control; and HG-Ins, high glucose + insulin.

Figure 3

(A, C and E) Immunoblots showing cyclin D1, phospho-p38, and phospho-ERK expression in MCF-7 cells and (B, D and F) immunoblots showing cyclin D1, phospho-p38, and phospho-ERK expression in MDA-MB-231 cells, after treatment with insulin (25 nM) for 30 min. Histograms represent the densitometrically quantified bar graphs of cyclin D1, p-p38, and p-ERK obtained after normalization with total p38, total ERK, and tubulin respectively. Results shown are representative of three different experiments. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ where a vs NG-C, b vs NG-Ins, and c vs HG-C. NG-C, normal glucose control; NG-Ins, normal glucose + insulin; HG-C, high glucose; and HG-Ins, high glucose + insulin.

(Fig. 3C, lane HG-Ins). On the other hand, we observed significant de-phosphorylation of p38 in MDA-MB-231 cells after insulin treatment (25 nM) for 30 min under a normal-glucose condition (Fig. 3D, lane NG-Ins). Moreover, high glucose and insulin treatment led to further de-phosphorylation of p38 (Fig. 3D, lane HG-Ins). This indicates that high glucose and insulin produces cellular stress and death in MCF-7 cells. By contrast, in MDA-MB-231 cells, it leads to cell survival and proliferation.

To further substantiate the above observation, we examined the phosphorylation status of ERK in both

the cell lines. ERK (or p42/44MAPK) is phosphorylated by the sequential activation of RAF1 and MEK1/2, thereby inducing cell survival and proliferation (Razidlo *et al.* 2004). It has been reported that ERK signaling down-regulates the expression of anti-apoptotic proteins and promotes survival of pancreatic cancer cells (Boucher *et al.* 2000). We observed de-phosphorylation of ERK by a high-glucose condition in MCF-7 cells (Fig. 3E, lane HG-C and HG-Ins). By contrast, in MDA-MB-231 cells, there was increased phosphorylation of ERK under a hyperglycemic condition (Fig. 3F, lane HG-C and HG-Ins). However, insulin could not produce significant change in ERK phosphorylation due to insulin treatment under normal- as well as high-glucose conditions. This is in line with the previous results and indicates that high glucose is inducing cell death in MCF-7 cells and proliferation in MDA-MB-231 cells.

Effect of high glucose and insulin on phosphorylation of GSK-3 β and NF- κ B in breast cancer cells

GSK-3 is a ubiquitously expressed serine/threonine kinase that exists in two isoforms, GSK-3 α and GSK-3 β . Under resting conditions, GSK-3 is active and is known to regulate GS, an enzyme that promotes glycogen deposition. Insulin results in inactivation of GSK-3 through phosphorylation of Ser 21 of GSK-3 α and Ser 9 of GSK-3 β . This leads to de-phosphorylation and activation of GS, resulting in increased glycogen synthesis (Patel *et al.* 2008). It has been reported that GSK-3 activity is increased in skeletal muscle and adipose tissue of obese rodents (Eldar-Finkel *et al.* 1999) as well as in skeletal muscles of patients with type II diabetes due to decreased insulin sensitivity (Nikoulina *et al.* 2000, Henriksen 2010). More recently, the literature also suggests a role of GSK-3 in diverse cellular processes including proliferation, differentiation, motility, and survival. Hence, we tried to investigate the role of GSK-3 β in high glucose- and insulin-induced proliferation in breast cancer cells. Our data clearly indicated that in MCF-7 cells, insulin treatment under a normal-glucose condition led to increased phosphorylation of GSK-3 β at Ser 9, which resulted in its inactivation (Fig. 4A, lane NG-Ins). Further increase in phosphorylation of GSK-3 β at Ser 9 was observed by treatment of insulin under a high-glucose condition (Fig. 4A, lanes HG-C and HG-Ins). Interestingly, in MDA-MB-231 cells, GSK-3 β was activated as observed by de-phosphorylation of GSK-3 β at Ser 9 by a high-glucose conditions (Fig. 4B, lanes HG-C and HG-Ins).

NF- κ B is a transcription factor, playing vital role in inflammation, cell proliferation, and survival. Recent

Figure 4

(A and C) Immunoblots showing phospho-GSK-3β and phospho-NF-κB expression in MCF-7 cells and (B and D) immunoblots showing phospho-GSK-3β and phospho-NF-κB expression in MDA-MB-231 cells, after treatment with insulin (25 nM) for 30 min. Histograms represent the densitometrically quantified bar graphs of p-GSK-3β and p-NF-κB obtained after normalization with total GSK-3β and total NF-κB. Results shown are representative of three different experiments. *P < 0.05, **P < 0.01, and ***P < 0.001 where a vs NG-C, b vs NG-Ins, and c vs HG-C. NG-C, normal glucose control; NG-Ins, normal glucose + insulin; HG-C, high glucose; and HG-Ins, high glucose + insulin.

reports indicate the inhibitory role of NF-κB in carcinogenesis (Perkins 2004, Chen & Castranova 2007). Its activity is regulated by GSK-3β, either positively or negatively, depending on the cell type being investigated (Grimes & Jope 2001). We showed that in MCF-7 cells, phosphorylation of NF-κB was increased by a high-glucose condition as well as by treatment of insulin under a high-glucose condition (Fig. 4C, lanes HG-C and HG-Ins). However, in MDA-MB-231 cells, we observed de-phosphorylation of NF-κB by high glucose and insulin treatment under normal- as well as high-glucose conditions (Fig. 4D, lanes HG-C and HG-Ins). Our results indicate that GSK-3β negatively regulated NF-κB activity in two different breast cancer cell lines. Additionally, we also provide evidence that high glucose is promoting carcinogenesis in MDA-MB-231 cells and inhibiting cell proliferation in MCF-7 cells.

Insulin- and high glucose-induced changes in phosphorylation of ERα in MCF-7 and MDA-MB-231 cells

It is known that ROS can act as subcellular messengers and modulate the gene regulatory and signal transduction pathways. Oxidative stress is known to regulate the

expression of ERα and ERβ (Tamir *et al.* 2002). As DCF-DA staining showed that high glucose and insulin generates ROS in MCF-7 and MDA-MB-231 cells, we investigated its effect on the phosphorylation of ERα. Our results show that a high-glucose condition increased the phosphorylation of ERα in MCF-7 cells, which are ERα-positive cell lines (Fig. 5A, lanes NG-Ins and HG-C). By contrast, we observed that in MDA-MB-231 cells (ERα-deficient cell lines), a high-glucose condition resulted in de-phosphorylation of ERα compared with a normal-glucose condition (Fig. 5B, lanes NG-Ins and HG-C). However, in both the cells, insulin treatment did not produce significant change in ERα expression under a high-glucose condition.

Alterations in post-translational modifications of histone H3 by hyperglycemia and insulin in breast cancer cells

Inducible covalent modifications of histone H3 have a major impact on cellular responses to various stimuli such as oxidative stress (Kabra *et al.* 2009). Histone H3 phosphorylation (Ser 10) is associated with induction of immediate early response genes, including proto-oncogenes, involved in differentiation, motility, and cancer (Thomson *et al.* 1999). Our results showed that phosphorylation of histone H3 decreased by insulin treatment under normal- as well as high-glucose conditions in MCF-7 cells (Fig. 6A, lanes NG-Ins and HG-Ins). In MDA-MB-231 cells, insulin treatment under a normal-glucose condition produced de-phosphorylation of histone H3. However, high glucose as well as insulin treatment under a high-glucose condition

Figure 5

(A and B) Immunoblots showing phospho-ERα in MCF-7 and MDA-MB-231 cells respectively after treatment with insulin (25 nM) for 30 min. Histograms represent the densitometrically quantified bar graphs of p-ERα obtained after normalization with total ERα. Results shown are representative of three different experiments. *P < 0.05 and ***P < 0.001 where a vs NG-C, b vs NG-Ins. NG-C, normal glucose control; NG-Ins, normal glucose + insulin; HG-C, high glucose; and HG-Ins, high glucose + insulin.

Figure 6

(A and B) Immunoblots showing the phosphorylation of histone H3 at serine 10; (C and D) immunoblots showing the acetylation of histone H3 in MCF-7 and MDA-MB-231 cells respectively after treatment with insulin (25 nM) for 30 min. Histograms represent the densitometrically quantified bar graphs of p-H3 and Ac-H3 in MCF-7 and MDA-MB-231 cells obtained after normalization with total histone H3. Results shown are representative of three different experiments. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ where a vs NG-C, b vs NG-Ins, and c vs HG-C. NG-C, normal glucose control; NG-Ins, normal glucose + insulin; HG-C, high glucose control; and HG-Ins, high glucose + insulin.

led to increased phosphorylation of histone H3 (Fig. 6B, lanes HG-C and HG-Ins). Histone H3 acetylation and de-acetylation modulates genes involved in cancer progression (Glozak & Seto 2007). Many literature findings indicate that acetylation of histone H3 blocks cell proliferation and induces apoptosis in cells (Vinodhkumar *et al.* 2008, Mathew *et al.* 2010, Shin *et al.* 2011, Anh *et al.* 2012). We provide evidence that insulin treatment under normal- as well as high-glucose conditions induced acetylation of histone H3 in MCF-7 cells (Fig. 6C, lane HG-Ins) and de-acetylation of histone H3 in MDA-MB-231 cells (Fig. 6D, lane HG-Ins).

Discussion

In this study, we provide the first evidence that high glucose promotes proliferation in MDA-MB-231 cells, whereas prevents it in MCF-7 cells by modulating the phosphorylation of p38, ERK, GSK-3 β , NF- κ B, and ER α . Further, it changes the expression of cyclin D1 and induces alterations in post-translational modifications of histone H3.

MCF-7 is a non-invasive, estrogen-dependent, early-stage breast cancer cell line, whereas MDA-MB-231 is an invasive, estrogen-independent, late-stage breast cancer cell line. MDA-MB-231 cells have higher aerobic glucose consumption rates (Gatenby & Gillies 2004) and higher expression of *GLUT1* (*SLC2A1*) gene (Laudanski *et al.* 2003) compared with MCF-7 cells, which results in greater invasiveness of the former. Therefore, we have utilized these two cell types with different metabolic phenotypes in order to study the effect of high glucose and insulin on cell proliferation.

ROS-induced oxidative stress results in induction of various biological responses that decide whether cells will undergo necrosis, senescence, apoptosis, or will survive and proliferate (Mates & Sanchez-Jimenez 2000). The extent of these responses will depend on the cellular genetic background, the types of the specific ROS involved, and the intensity and duration of the oxidative stress (Pelicano *et al.* 2004). It has been reported that ROS is involved in oncogenic transformation (Behrend *et al.* 2003). Severe oxidative stress induced by high glucose and insulin in MCF-7 cells might result in cell death, whereas that in MDA-MB-231 cells, which have a more aggressive phenotype, may undergo proliferation. This can be explained if we assume that MDA-MB-231 cells are adapting to oxidative insult induced by high glucose and insulin, thereby developing an enhanced endogenous antioxidant capacity. Sub-lethal oxidative stress can produce resistance to apoptosis and promote cell proliferation (Burdon 1995). Literature reports indicate that there is upregulation of glucose metabolism in metastatic cancer cells, which results in adaptive response to acid-induced apoptosis and proliferation of cells (Gatenby & Gillies 2004, Gillies *et al.* 2008). MTT and BrdU assays further prove that high glucose prevented proliferation of MCF-7 cells and promoted it in MDA-MB-231 cells.

MAPKs are known to be involved in cell growth, differentiation, and apoptosis. It has been reported that increased glucose concentration generates intracellular ROS, which activates p38, resulting in apoptosis in podocytes (Susztak *et al.* 2006). Further, high glucose-induced phosphorylation of p38 in human endothelial cells leads to cell death (Nakagami *et al.* 2001). ERK1/2 activity is associated with invasive and metastatic properties (Whyte *et al.* 2009); its activation results in desensitization of pore opening and increased resistance to death stimuli, providing advantage to tumor cells (Rasola *et al.* 2010). Our results show that cellular stress under a high-glucose condition in MCF-7 cells results in activation of p38 and de-phosphorylation of ERK, thereby leading to cell death. By contrast, in MDA-MB-231 cells, inactivation of p38 and

phosphorylation of ERK suggests that these more aggressive, metastatic cells adapt to high glucose-induced oxidative stress and undergo proliferation. As the duration of insulin treatment in this study was 30 min, we failed to observe significant changes due to insulin treatment. Weichhaus *et al.* (2012) had also suggested that insulin plays a role in MDA-MB-231 cell metabolism, but not in increasing cell proliferation and enhancing cell cycle progression.

Cyclin D1 is an important regulator of cell cycle progression and its over-expression has been linked to the development and progression of cancer (Sutherland & Musgrove 2002). Decreased cyclin D1 expression in MCF-7 cells and its increased expression in MDA-MB-231 cells by a high-glucose condition further confirm our preceding results. Expression of cyclin D1 is regulated by GSK-3 β (Takahashi-Yanaga & Sasaguri 2008), which is involved in the process of tumorigenesis; its over-expression drives the cells into S phase and facilitates cell proliferation (Luo 2009). Phosphorylation of GSK-3 β at tyrosine 216 results in activation of GSK-3 β , whereas phosphorylation at serine 9 inhibits its activity (Fang *et al.* 2000). Hyperglycemia inactivates GSK-3 β in MCF-7 cells, whereas it activates it in MDA-MB-231 cells. As GSK-3 β activity is increased under a high-glucose condition in MDA-MB-231 cells and also in skeletal muscles of patients with diabetes (Nikoulina *et al.* 2000), our result advocates that there is increased susceptibility of diabetic patients toward progression of metastatic breast cancer.

NF- κ B is critical for regulation of gene expression in response to inflammatory stimuli and it has both survival and apoptotic functions. It has been linked to the p53 tumor suppressor pathway leading to apoptosis (Ryan *et al.* 2000). NF- κ B inhibits tumor growth in the early stages, but, as further mutations occur in the tumor suppressor genes, its oncogenic functions become dominant, contributing to tumorigenesis (Perkins 2004). We show that activation of NF- κ B by high glucose and insulin treatment in MCF-7 cells results in an inflammatory response, which may be responsible for activation of pathways responsible for cell death. Moreover, its inactivation in MDA-MB-231 cells under similar conditions suggests that inflammatory pathways are not activated in these cells and hence there is no cell death. Instead, it promotes proliferation and carcinogenesis in late-stage, metastatic breast cancer cells.

GSK-3 β plays a role in ligand-independent activation of ER α and leads to its phosphorylation at serine 118 (Medunjanin *et al.* 2005). Estrogen and ERs promote proliferation and survival of breast cancer cells (Tan *et al.* 2009). Moreover, oxidative stress is also reported to influence the regulation of ER (Tamir *et al.* 2002) and

induces its phosphorylation at serine 118 and 167 (Weitsman *et al.* 2009). Earlier, Chakrabarti & Davidge (2009) had demonstrated that high glucose-induced oxidative stress alters estrogen-mediated regulation of ER α and ER β expression in human endothelial cells. A recent report showed that estrogen modulates glucose metabolism in breast cancer cells by upregulating glycolysis under a high-glucose condition (O'Mahony *et al.* 2012). Under resting conditions, GSK-3 β and ER α forms a complex in the cytoplasm. GSK-3 β phosphorylation at serine 9 dissociates the complex and subsequently ER α is phosphorylated at ser 118 (Medunjanin *et al.* 2005). We infer that in MCF-7 cells, high glucose-induced phosphorylation of GSK-3 β at serine 9 leads to increased phosphorylation of ER α . By contrast, high glucose-induced de-phosphorylation of GSK-3 β in MDA-MB-231 cells results in de-phosphorylation of ER α . It has been reported that ER α activation significantly decreases ERK phosphorylation and reduces proliferation of VSMC under a high-glucose condition (Ortmann *et al.* 2011). We demonstrate that a high-glucose condition activates ER α and de-phosphorylates ERK in MCF-7 cells, subjecting them to apoptotic cell death, but not in MDA-MB-231 cells.

Several reports indicate that oxidative stress leads to covalent modifications of histone H3 (Rahman 2002, Monks *et al.* 2006, Tikoo *et al.* 2008, Rajendrasozhan *et al.* 2009). Alterations in modifications of histones have been linked to deregulated expression of many genes, with important roles in cancer development and progression (Kurdistani 2007). Phosphorylation of histone H3 at serine 10 induced neoplastic cell transformation in JB6 Cl41 mouse epidermal cells (Choi *et al.* 2005). Insulin treatment under a high-glucose condition induces de-phosphorylation and hyper-acetylation of histone H3 in MCF-7 cells, and phosphorylation and de-acetylation of histone H3 in MDA-MB-231 cells, indicating cell death in the former and enhanced proliferation and neoplastic transformation of aggressive, metastatic cells MDA-MB-231.

In conclusion, high glucose modulates cyclin D1 expression, phosphorylation of p38, ERK, GSK-3 β , NF- κ B, ER α , and modifications of histone H3, thereby preventing proliferation in MCF-7 and promoting it in MDA-MB-231 breast cancer cells. This suggests that the two cancer cell lines are behaving differentially with respect to glucose metabolism. We suggest that a diet high in glycemic load may lead to progression of hormone-independent, metastatic breast cancer cells. Better understanding of the mechanisms involved in the association of hyperglycemia/hyperinsulinemia and breast cancer could help in prevention of the progression of the disease.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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