

RESEARCH ARTICLE

PKC δ -dependent Activation of the Ubiquitin Proteasome System is Responsible for High Glucose-induced Human Breast Cancer MCF-7 Cell Proliferation, Migration and Invasion

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Abstract

Type 2 diabetes mellitus (T2DM) has contributed to advanced breast cancer development over the past decades. However, the mechanism underlying this contribution is poorly understood. In this study, we determined that high glucose enhanced proteasome activity was accompanied by enhanced proliferation, migration and invasion, as well as suppressed apoptosis, in human breast cancer MCF-7 cells. Proteasome inhibitor bortezomib (BZM) pretreatment mitigated high glucose-induced MCF-7 cell growth and invasion. Furthermore, high glucose increased protein kinase C delta (PKC δ)-phosphorylation. Administration of the specific PKC δ inhibitor rottlerin attenuated high glucose-stimulated cancer cell growth and invasion. In addition, PKC δ inhibition by both rottlerin and PKC δ shRNA significantly suppressed high glucose-induced proteasome activity. Our results suggest that PKC δ -dependent ubiquitin proteasome system activation plays an important role in high glucose-induced breast cancer cell growth and metastasis.

Keywords: High glucose - ubiquitin proteasome system - protein kinase C δ - breast cancer

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Introduction

Breast cancer is one of the most common cancers and is the most common cause of cancer mortality in women worldwide (Jemal et al., 2011). Several type 2 diabetes mellitus (T2DM) risk factors, such as old age and obesity, are associated with breast cancer (Wolf et al., 2005). Conversely, T2DM is linked to increasing breast cancer incidence and mortality (Liao et al., 2011). Meta-analysis reveals that breast cancer risk in patients with T2DM is increased by 20-40% (Larsson et al., 2007). Diabetic women have a higher risk of dying from breast cancer than those without diabetes (Coughlin et al., 2004). One possible mechanism is that the hyperglycemia in T2DM patients may promote cancer progression, because the neoplastic cells could use glucose for proliferation via the pentose phosphate pathway (Warburg, 1956; Dang and Semenza, 1999). However, the detailed mechanism(s) remains unknown.

The ubiquitin proteasome system (UPS) and the autophagy-lysosome pathway are two main routes of the intracellular protein-degradation (Goldberg, 2003; Rubinsztajn, 2006). Current studies demonstrate that

impaired protein degradation promotes the pathogenesis of some diseases including cancers. UPS deregulation plays a key role in cell cycle progression, proliferation, apoptosis, angiogenesis and cell signaling pathways that contribute to cancer development (Ciechanover and Schwartz, 1994; Hochstrasser, 1995; Ciechanover et al., 2000; Orłowski and Dees, 2003). Thus, it is necessary to keep the UPS functional for normal and neoplastic cell survival. It is interesting that diabetes increases ubiquitin expression but does not alter proteasome chymotrypsin-like peptidase activity in the heart and skeletal muscle (Liu et al., 2000). We do not know whether the UPS is involved in high glucose-induced carcinogenesis regulation.

In the present study, we determined that high glucose-induced proliferation, migration, and invasion were attenuated by treatment with the UPS inhibitor bortezomib (BZM) in human breast cancer MCF-7 cells. We also demonstrated that both the PKC δ specific inhibitor rottlerin and PKC δ shRNA suppressed UPS activity. Our data suggest that PKC δ mediated high glucose-induced UPS activation and that targeting the UPS and reducing serum glucose levels may represent a novel therapeutic strategy for breast cancer patients.

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Materials and Methods

Cell culture

Human breast cancer MCF-7 cells (ATCC, Manassas, USA) were cultured in RPMI 1640 (Gibco BRL, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) (HyClone, Logan, UT, USA) and 1% penicillin/streptomycin (Invitrogen, Grand Island, NY) at 37 °C in a humidified atmosphere containing 5% CO₂.

Proliferation assay

The MTT assay was used to evaluate cell proliferation. The thiazolyl blue tetrazolium bromide (MTT) (Amresco, Solon, OH, USA) was dissolved in phosphate buffered saline (PBS) at a concentration of 5 mg/ml, filtered, and stored at 4 °C. Cells were seeded into a 96-well plate, washed three times with PBS and cultured in RPMI 1640 without FBS and glucose. After 12 hours, cells were treated with the indicated glucose concentrations of (Sigma, St. Louis, MO, USA), bortezomib (BZM, Santa Cruz, Texas, USA) and/or rottlerin (Sigma) for 48 hrs. The osmotic pressure was balanced by D-Mannitol (Sigma). For the proliferation assay, 20 µl MTT was added into each well. An ELISA plate reader (Biotek, Winooski, Vermont, USA) was used to measure the optical density at 490 nm.

TUNEL assay

The TUNEL assay was performed using an In Situ Cell Death Detection Kit (Roche, Basel, Switzerland) following the manufacturer's instructions. Cells that were cultured in RPMI 1640 containing 10% FBS were used as a negative control. Images were taken with the Olympus FluoView FV1000 Confocal Microscope.

Wound healing assay

MCF-7 cells were grown to confluent monolayers on 6-well plates and a pipette tip was used to create linear scratch wounds. Mitomycin C (Amresco) was used to inhibit cell proliferation. Wound images were taken with a digital camera mounted on light microscope. The wound gap widths were measured using Image J software.

Transwell assay

The upper chamber of each 8.0 µm pore size Transwell apparatus (Corning, NY, USA) was coated with Matrigel (BD Biosciences, San Jose, CA). MCF-7 cells were added to the upper chamber at a density of 10⁶ cells/ml (100 µl per chamber) and incubated for 24 hrs followed by removal of the cells that remained in the top chamber with cotton swabs. Cells that penetrated to the lower membrane surface were fixed in 4% paraformaldehyde, stained with crystal violet, and counted under a microscope.

Plasmids and virus infection

PKCδ shRNA lentiviruses were obtained from Santa Cruz Biotechnology, RFP adenoviruses and specific ubiquitin-proteasome reporter GFPu were kind gifts from Dr. Xuejun (XJ) Wang (The University of South Dakota). MCF-7 cells were infected with PKCδ shRNA lentiviruses (MOI: 50), GFPu adenoviruses (MOI: 20), or RFP adenoviruses (MOI: 20) for 6 hrs and were then

cultured in RPMI 1640 containing 5.5 mM glucose for 24 hrs for experiments. Cell lysates were collected and Western blots were performed to detect protein expression using specific antibodies.

Western blots

Cells were collected with lysis buffer after being washed three times with ice-cold PBS. Lysates were boiled in SDS loading buffer for 10 min then cleared by centrifugation (14,000 rpm, 10min, 4 °C). The proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and detected with specific antibodies.

Proteasomal peptidase activity assay

Proteasomal peptidase activities in MCF-7 cells were measured using described methods (Naujokat et al., 2007). Briefly, cells were washed twice with cold PBS and lysed with cytosolic extraction buffer (50 mmol/l HEPES buffer, pH 7.5 containing 20 mmol/l KCl, 5 mmol/l MgCl₂, 2 mmol/l ATP, 1 mmol/l DTT, 0.025% Digitonin). The lysate was centrifuged at 10,000 × g for 30 min at 4 °C and the BCA assay was performed to measure protein concentration. Suc-LLVY-AMC, ZLLE-AMC, and Ac-RLR-AMC were used to measure chymotrypsin-like activity, caspase-like activity, and trypsin-like activity, respectively. For assay specificity, 20 µM proteasome inhibitor MG132 was incubated with the extract. After 30 min incubation at 37°C, the fluorescence intensity was read (excitation, 380 nm; emission, 440 nm) using a fluorescence spectrometer (SpectraMax M2, Molecular Devices, Sunnyvale, CA, USA).

Statistical analysis

Results are represented as the mean ± SEM. The quantification of the relative increase in protein expression and phosphorylation was performed using NIH Scion Image software and was normalized with the control protein expression in each experiment. The figures were representative of at least three independent experiments with similar results. Differences between mean values were examined using the paired Student's t-test. *p* < 0.05 was considered to be statistically significant.

Results

Effects of high glucose on MCF-7 cell growth, migration, and invasion

To investigate the effects of high glucose on cancer cell growth, MCF-7 cells were incubated in 5.5 mM, 11 mM, or 22 mM glucose for 48 hrs. MTT and TUNEL assays were performed to measure cell proliferation and apoptosis. As demonstrated in Figure 1A, glucose enhanced MCF-7 cell proliferation in a concentration-dependent manner. Accordingly, high glucose suppressed MCF-7 cell apoptosis (Figure 1B). We then studied the effects of high glucose on MCF-7 cell migration and invasion using wound healing and transwell assays as described in the materials and methods section. Mitomycin C was administered to inhibit cell proliferation. We determined that relative wound closure (Figure 1C) and the number

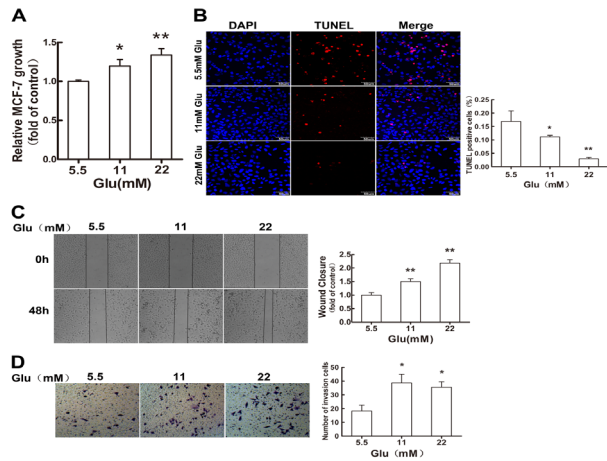


Figure 1. High Glucose Enhanced MCF-7 Cell Growth, Migration, and Invasion. (A) Effects of high glucose on cell proliferation. (B) Effects of high glucose on apoptosis. (C) Effects of high glucose on wound healing. (D) Effects of high glucose on cell invasion. * $p < 0.05$, ** $p < 0.01$ vs. the 5.5 mM Glu: glucose

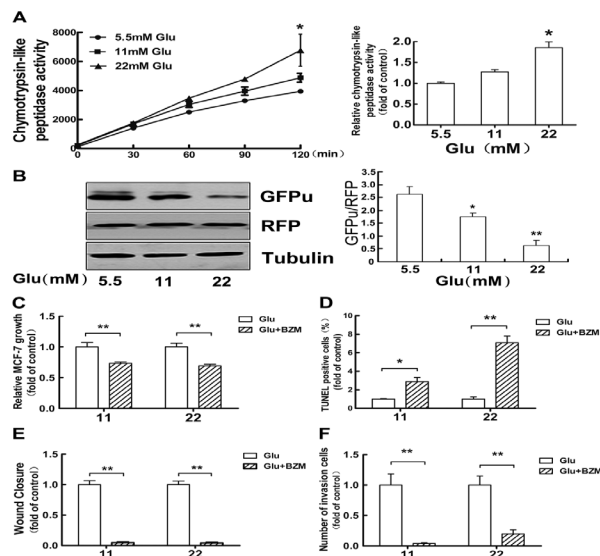


Figure 2. Ubiquitin Proteasome System (UPS) Activation Contributed to High Glucose-induced MCF-7 Cell Growth, Migration, and Invasion. (A) Effects of high glucose on proteasome activity. (B) Effects of high glucose on GFPu protein expression and the GFPu/RFP ratio. (C) Effects of BZM on cell proliferation. (D) Effects of BZM on cell apoptosis. (E) Effects of BZM on wound healing. (F) Effects of BZM on cell invasion. * $p < 0.05$, ** $p < 0.01$ vs. the 5.5 mM group or untreated groups. Glu: glucose, BZM: bortezomib

of invading cells (Figure 1D) was significantly increased in cells that had been treated with 11 mM and 22 mM glucose for 48 hrs.

Effects of the ubiquitin-proteasome system (UPS) activity on high glucose-treated MCF-7 cell growth, migration, and invasion

During the past decade, it has been suggested that the ubiquitin-proteasome system is involved in cancer development, and targeting this pathway with proteasome inhibitors such as bortezomib (BZM) is a potential strategy for cancer treatment (Dees and Orlowski, 2006; Landis-

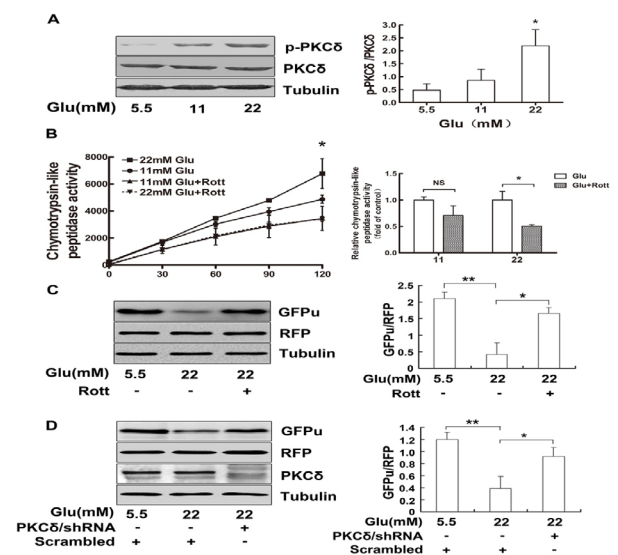


Figure 3. PKC δ Upregulated UPS Activity in High Glucose-treated MCF-7 Cells. (A) Effects of high glucose on PKC δ phosphorylation. (B) Effects of rottlerin on proteasome activity. (C) Effects of rottlerin on GFPu protein expression and the GFPu/RFP ratio. (D) Effects of PKC δ knockdown on GFPu protein expression and the GFPu/RFP ratio. NS=not significant, * $p < 0.05$, ** $p < 0.01$ vs. the 5.5 mM group or untreated groups. Glu: glucose, Rott: rottlerin.

Piowar et al., 2006; Sato et al., 2008). To figure out whether glucose regulated UPS activity, we first assessed proteasome peptidase activity in MCF-7 cells that had been treated with the indicated glucose concentrations for 48 hrs. As demonstrated in Figure 2A, chymotrypsin-like peptidase activity was increased in a glucose concentration-dependent manner. However, trypsin-like and caspase-like proteasome peptidase activities remained unchanged (data not shown). To confirm that the UPS was specifically activated by high glucose, MCF-7 cells were coinfecting with RFP (MOI: 20) and GFPu adenoviruses (MOI: 20) followed by incubation in the presence of 5.5, 11, or 22 mM glucose for 48 hrs. Western blots were performed to detect RFP and GFPu protein expression. We determined that high glucose significantly decreased GFPu protein expression and the GFPu to RFP ratio (Figure 2B). To determine the effects of UPS on high glucose-induced cancer cell growth, migration, and invasion, MCF-7 cells were pretreated with 50 nM BZM for 1 hr followed by incubation in the presence of 11 or 22 mM glucose for another 48 hrs. We determined that BZM pretreatment significantly mitigated high glucose-induced cell growth (Figure 2C), enhanced apoptosis (Figure 2D), suppressed wound closure (Figure 2E) and reduced invasion of MCF-7 cells (Figure 2F).

Effects of PKC δ on UPS activity in high glucose-treated MCF-7 cells

To elucidate the mechanism by which UPS activity was upregulated, MCF-7 cells were cultured in RPMI 1640 containing 5.5, 11, or 22 mM glucose for 48 hrs. PKC isoform phosphorylation was detected by Western blot using specific antibodies. Our data demonstrated that PKC δ phosphorylation was significantly enhanced by high glucose treatment (Figure 3A). Other PKC

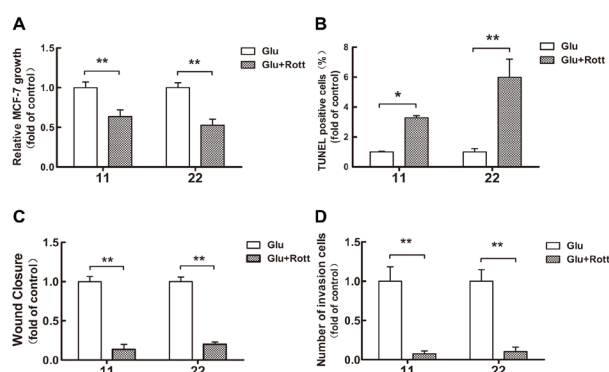


Figure 4. PKC δ Inhibition Suppressed High Glucose-induced MCF-7 Cell Growth, Migration, and Invasion.

(A) Effects of rottlerin on cell proliferation. (B) Effects of rottlerin on apoptosis. (C) Effects of rottlerin on wound healing. (D) Effects of rottlerin on cell invasion. * $p < 0.05$, ** $p < 0.01$ vs. the untreated groups. Glu: glucose, Rott: rottlerin

isoform phosphorylation remained unchanged (data not shown). To investigate the effect of PKC δ on UPS activity, MCF-7 cells were pretreated with the PKC δ specific inhibitor rottlerin (250 nM) for 1 hr followed by treatment with 11 or 22 mM high glucose for another 48 hrs, and proteasomal peptidase activity was measured. As demonstrated in Figure 3B, rottlerin pretreatment significantly decreased chymotrypsin-like peptidase activity. To confirm the effects of PKC δ on the UPS, we investigated whether PKC δ activity affected specific proteasome substrate GFPu expression. RFP and GFPu were overexpressed in MCF-7 cells by coinfecting cells with RFP and GFPu adenoviruses (MOI: 20). PKC δ was inhibited by pretreating the cells with rottlerin (250 nM) for 1 hr. PKC δ was knocked down in MCF-7 cells by PKC δ shRNA lentivirus infection (MOI: 100). Cells were then incubated in the presence of 5.5 or 22 mM glucose for another 48 hrs. We determined that both rottlerin (Figure 3C) and PKC δ shRNA (Figure 3D) restored GFPu protein expression and the GFPu to RFP ratio.

Effects of PKC δ inhibition on high glucose-treated MCF-7 cell growth, migration, and invasion

To confirm the functional consequences of PKC δ inhibition on cancer cell growth, migration, and invasion, MCF-7 cells were incubated with 11 or 22 mM glucose for 48 hrs. Cell growth, migration, and invasion were assessed by MTT, TUNEL, wound healing, and transwell assays, respectively. The results demonstrated that the PKC δ inhibitor rottlerin significantly reduced cell proliferation (Figure 4A), increased apoptosis (Figure 4B), suppressed wound-closure (Figure 4C) and reduced the number of invading cells (Figure 4D).

Discussion

In recent years, type 2 diabetes mellitus (T2DM) has been suggested to be a negative prognostic factor for breast cancer, especially in premenopausal women (Liao et al., 2010). One potential mechanism for this correlation is that hyperglycemia or high glucose benefits advanced tumor propagation via the Warburg effect (Hauptmann

et al., 2005; Gillies and Gatenby, 2007; Kroemer and Pouyssegur, 2008). Our finding in the present study revealed that high glucose significantly increased human breast cancer MCF-7 cell growth, migration, and invasion, which supports this theory (Figure 1).

The UPS is a main route for protein degradation that plays an important role in maintaining protein homeostasis and quality control. Previous studies have demonstrated that the UPS is involved in regulating some very important cellular processes such as signal transduction, growth, proliferation, differentiation and apoptosis. Abnormal UPS function can result in a malignant cellular phenotype. Thus, proteasome inhibition is an attractive target for novel anticancer therapy (Marfella et al., 2007; Wu et al., 2010; Bedford et al., 2011; Driscoll and Woodle, 2012; Micel et al., 2013). Although the role of the UPS in type 2 diabetes pathogenesis remains largely unexplored, some studies have suggested that UPS activation is responsible for the diabetes-induced insulin signaling impairment, pancreatic beta-cell dysfunction, and cardiovascular complications (Wing, 2008; Marfella et al., 2009; Kirk-Ballard et al., 2013). In contrast, Liu et al. demonstrated that acute diabetes did not increase proteasome chymotrypsin-like peptidase activity in the heart and skeletal muscle (Liu et al., 2000). Selected UPS genes and proteasome activity were downregulated in T2D islets and beta cell fractions (Bugliani et al., 2013). These data highlight the importance of the UPS in cancer and diabetes development. In the present study, we determined that chymotrypsin-like peptidase activity was significantly increased in high glucose-treated MCF-7 cells (Figure 2A). Consistent with this result, GFPu protein expression was reduced (Figure 2B). Because GFPu is a specific ubiquitin proteasome system substrate that could be used as a dynamic reporter of UPS activity in vivo (Bence et al., 2001), our results gave evidence to support the possibility that high glucose enhanced breast cancer cell UPS activity. Our results also determined that proteasome inhibitor bortezomib (Cvek and Dvorak, 2011) pretreatment significantly mitigated cell proliferation (Figure 2C), increased apoptosis (Figure 2D), attenuated wound closure (Figure 2E) and reduced the number of invading cells (Figure 2F), suggesting that high glucose-induced MCF-7 cell growth, migration, and invasion are mediated by the UPS.

The remaining question is how does high glucose activate the UPS? We determined that high glucose specifically induced PKC δ phosphorylation (Figure 3A). This finding is consistent with previous studies that demonstrated that high glucose activated PKCs. A possible mechanism for inducing PKCs activation is increased reactive oxygen species (ROS), which are generated in response to hyperglycemia (Srivastava, 2002; Wu, 2006; Sasaki and Inoguchi, 2012). Our results also demonstrated that specific PKC δ inhibitor rottlerin pretreatment significantly suppressed high glucose-induced chymotrypsin-like peptidase activity (Figure 3B). The high glucose-mediated GFPu expression reduction was restored by both rottlerin administration and PKC δ knockdown (Figure 3C and Figure 3D). These findings strongly suggest that activated PKC δ contributes to

increased proteasome activity (Smith et al., 2004). PKCs are crucial regulators of cancer cell growth, migration, and invasion (Masur et al., 2011). Our data revealed that rottlerin-mediated PKC δ inhibition suppressed MCF-7 growth, migration, and invasion (Figure 4), consistent with earlier studies demonstrating that PKC δ can enhance malignant mammary cell proliferation, metastasis and survival (Kiley et al., 1999; Grossoni et al., 2007).

In summary, our results demonstrated for the first time that PKC δ -dependent UPS activation upregulated MCF-7 cell growth and invasion after high glucose treatment. Characterizing the mechanism by which PKC δ regulated the UPS and its pathophysiological consequences will provide important information regarding breast cancer diagnosis and therapy.

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