Insulin-induced GPX4 Expression in Breast Cancer Cells

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Abstract

Phospholipid hydroperoxide glutathione peroxidase (GPX4) protein is an anti-oxidative enzyme that can directly reduce both phospholipids and cholesterol-hydroperoxides located in cell membranes and lipoproteins. In this study we describe the induction of glutathione peroxidase 4 (GPX4) protein by insulin in MCF-7 breast cancer cells. Herein we report that insulin elicited a time-dependent increase in GPX4 expression in MCF-7 breast cancer cells. Additionally, insulin triggered a rapid and transient increase of intracellular reactive oxygen species (ROS) levels, and that insulin-induced increase on ROS levels was inhibited by pretreatment with N-acetyl-L-cysteine (NAC). Pretreatment of cells with LY294002, a pharmacological inhibitor of phosphatidylinositol 3-kinase (PI3K) inhibitor, suppressed the insulin-augmented GPX4 expression, which suggests that the PI3K/Akt pathway mediates this effect of insulin. Collectively, these data suggest that insulin can modulate the GPX4 protein level by activation of PI3K/Akt via the generation of ROS.

Key words: Insulin, reactive oxygen species, glutathione peroxidase, breast cancer

Introduction

Insulin is a polypeptide hormone that regulates glucose, lipid, and protein metabolism and promotes cell growth and differentiation. The insulin receptor tyrosine kinase, on ligand binding, initiates multiple signaling cascades, including activation of the phosphatidylinositol 3-kinase (PI3K) and its downstream effectors.[1] This pathway is a key signal transducer of many growth factors and cytokines and has been implicated in the regulation of cell growth, cell migration and cell survival.[2] Recent work has been shown a direct link between the PI3K/Akt pathway and induction of reactive oxygen species (ROS). It has been suggested that a low concentration of ROS can exert important physiological roles in cellular signaling and proliferation.[3]

Several growth factors (platelet-derived growth factor, epidermal growth factor, and nerve growth factor), cytokines (IL-1 and TNF-α) and hormone (insulin) are known to stimulate cell growth via the generation of ROS in many target cells.[4] Previous studies demonstrated that ROS accumulation induced by TNF alpha,[5] PDGF,[6] or VEGF7) was suppressed when PI3K activity/activation was blocked by pharmacological or transfectional means. Therefore, the broad spectrum of responses to oxidants in proliferating cells suggests a new paradigm for oxidative stress.[3] To control the balance between the production and

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removal of oxygen radicals, cells contain a variety of antioxidant enzymes such as glutathione peroxidase (GPX), superoxide dismutase, catalase, and glutathione-S-transferase. Among the different enzymes, GPX4 is a well known anti-oxidative enzyme that can directly reduce peroxidized phospholipids, fatty acids, and cholesterol in membranes.[8] Some supporting evidences suggest that up-regulation of GPX4 expression and subsequent increase in the GPX4 activity may confer adaptive survival response to oxidative stress. The GPX4-overexpressing RBL2H3 cells are reported to be resistant to necrotic and apoptotic cell death caused by various oxidative stress.[9] In vivo model for this effect have shown that tansgenic mice overexpressing GPX4 are protected against oxidative stress-induced apoptosis.[10] In this study, we analyzed the effect of insulin on expression of the antioxidant enzyme, GPX4. In addition, we examined the mechanisms of how insulin regulates the expression of GPX4 protein in MCF-7 cells.

Materials and Methods

1. Reagents and Cell Culture

Insulin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrozolum bromide (MTT), ICI 182,780 [N-n-butyl-N-methyl-11(3,17-dihydroxyoestra-1,3,5-trien-7-yl) undecamide] and antibody to β-actin were obtained from Sigma-Adrich Co. (St. Louis, MO, USA). LY294002 [2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one], PD98059 (2'-amino-3'-methoxyflavone), and Gö6976 [12-(2-cyanoethyl)-tetrahydro-13-methyl-oxoindolopyrrolocarbazole] were from Calbiochem. (La Jolla, CA, USA). The rabbit polyclonal antibodies specific to Akt and phospho-Akt (Ser473) were from Cell Signaling (Bevery, MA, USA). Goat anti-rabbit IgG-HRP antibody, and enhanced chemiluminescence (ECL) system was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cell culture media and reagents was purchased from Life Technologies (Grand Island, NY, USA). The human mammary adenocarcinoma cell line, MCF-7, obtained from Korean Cell Line Bank (Seoul, Korea), was used throughout the study. The cells grew in a monolayer culture in DMEM supplemented with 5% fetal calf serum (FCS), 1 mM glutamine, 100 units of penicillin/ml and 100 ug of streptomycin/ml. Cells were grown to 70% confluence, and the medium was changed to phenol red-free DMEM with 5% charcoal-stripped calf serum (CCS). Cells were maintained in this medium for 24 h before treatment.

2. MTT assay

MCF-7 cells were seeded in 24-well plates in DMEM supplemented with 5% FCS. Cells were grown to 70% confluence, and the medium was changed to phenol red-free DMEM with 5% CCS. Cells were maintained in this medium for 24 h followed by recombinant human insulin treatment for 24, 48 and 72 h. MTT (final 0.1 mg/ml) was added to the culture medium and cells were incubated for additional 4 h. After removal of the medium, the formazan crystals formed by the reduction of MTT by mitochondrial dehydrogenases in living cells were solubilized in 500 ul of DMSO and measured spectrophotometrically at 555 nm. The results were expressed as the percentage based on the ratio of absorbances of treated cells to controls (100%).

3. Measurement of intracellular Reactive oxygen species

Intracellular ROS levels were measured using DCF-DA (Wang and Joseph, 1999). Briefly, MCF-7 cells were pretreated with vehicle (media) or NAC (10 mM) for 1 h prior to incubation with insulin (50 nM) for the indicated times, after which the cells were loaded with 10 μM of DCFH-DA (Molecular Probes, Oregon) for 20 min. Next, the cells were harvested and washed twice with 1X PBS to remove all excess DCF-DA that had not penetrated the cells. The relative fluore- scence was measured at the excitation and emission wavelengths of 485 and 530 nm, res- pectively, using a multi-well fluorescence plate reader.

4. Western blot analysis for GPX4 expression

Cell lysates were prepared using a buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40). Briefly, proteins (40 μg per lane) were separated on 10% Tris-glycine SDS-polyacrylamide gels and then electrophoretically transferred to Immuno -Blot PVDF membranes. The membranes were then incubated for 1 h at room temperature with a 1:500 dilution of goat anti-GPX4 polyclonal antibody. Next, horseradish peroxidase-conjugated secondary antibody was applied at a dilution of 1:8000. Anti--actin antibody was used as a loading control and the signal was visualized using an ECL detection kit.

Results and Discussion

To determine the potency of insulin-mediated cell proliferation, MCF-7 cells were treated with recombinant human insulin for 3 day. Insulin induced cell growth in a dose- and time-dependent manner (Fig. 1). Several studies have shown that insulin stimulates the production of intracellular ROS, which can be involved in signaling pathways.[11] Thus, to evaluate whether

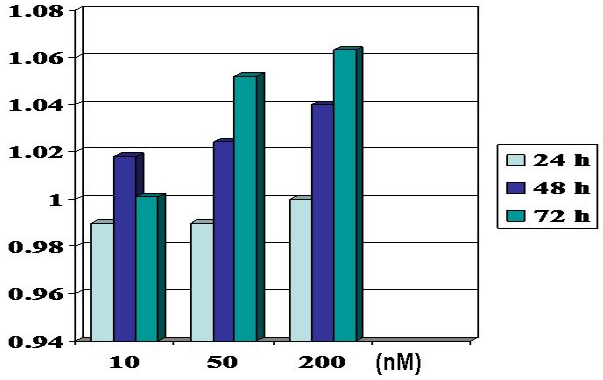

Fig. 1. Insulin-induced cell growth. MCF-7 cells were plated in 5% CCS-containing DMEM for 24 h followed by treatment with insulin (10-200 nM) for 72 h and harvested for viability. The percentage of viable cells was determined by MTT assay as precisely described in Material and Methods.

insulin can trigger the production of intracellular ROS and to explore the link between insulin-induced ROS generation and GPX4 expression, MCF-7 cells were treated with insulin for various periods of time and measured the intracellular ROS levels using the fluorophore DCF-DA. As shown in Fig. 2, the intracellular ROS levels increased as early as 10 min after stimulation with insulin and then gradually declined until 60 min.

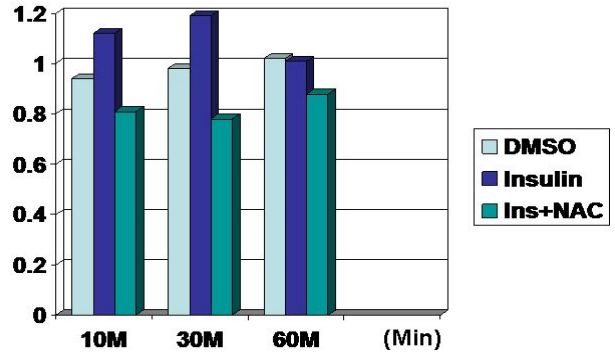

Fig. 2. Modulation of the GPX4 protein expression by insulin-induced ROS. Time course of ROS generation induced by insulin. MCF-7 cells were treated with

vehicle (media) or NAC (10 mM) for 1 h prior to incubation with insulin (50 nM) for the indicated times. Intracellular ROS levels were then measured using the redox-sensitive dye DCF-DA as described in the Material and Method

However, pre-treatment with antioxidants, NAC, significantly reduced insulin-induced ROS levels in the cells.

Next, to determine the role of the signaling pathways in the induction of GPX4, we used pharmacological inhibitors that block a specific signaling pathway; PD98059 for MEK1/2, Go for protein kinase or Ly294002 for PI3K. As shown in Fig. 3, LY294002 effectively inhibited expression of GPX4 protein induced by insulin.

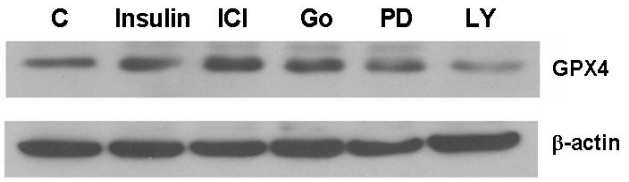

Fig. 3. Effects of various chemical inhibitors on GPX4 level. (A) MCF-7 cells were treated with vehicle (media) or with ICI 182,780 (5 uM), Gö6976 (2 uM), PD098059 (50 uM), and LY294002 (25 uM) for 1 h before an overnight incubation with insulin. Cell lysates (40 ug) were prepared for Western blotting using anti-GPX4 antibody. β-actin was used as a loading control.

On the other hand, Go and PD98059 had no significant effect on the induction of GPX4 by insulin, suggesting that ERK1/2 is not likely to be involved in its expression. Interestingly, regardless of pre-incubation with the ER antagonist, ICI 182,780, up-regulation of the GPX4 expression following insulin treatment was not clearly blocked under the preincubation with the ER antagonist, ICI 182,780. Taken together, these results imply that signaling mediated by PI3K/Akt can play a significant role in GPX4 induction by insulin in MCF-7 cells. To also examine whether PI3K/Akt signaling is involved in insulin-induced down-regulation of GPX4, Akt phosphorylation was tested. insulin induced slightly induced phosphorylation of Akt(Ser473) after 30 min. The phosphorylation of Akt remained at elevated levels for at least 2 h following insulin exposure. No change in total Akt level was observed (Fig. 4). Taken together, these results indicate that the Akt pathway participates in the induction of GPX4 induction in MCF-7 cells.

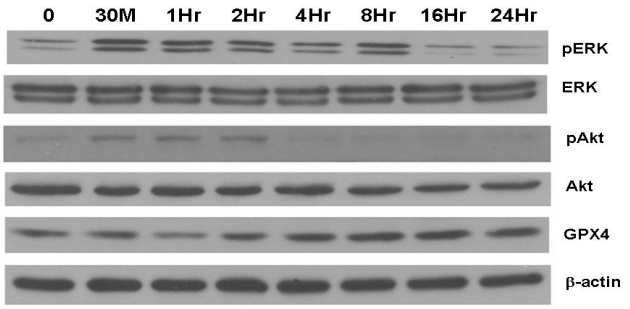

Fig. 4. Insulin-induced up-regulation of the GPX4 protein. MCF-7 cells were treated with vehicle (media) or insulin (50 nM) for the indicated times (A). Cell lysates (40 ug) were then analyzed by Western blot analysis to determine the level of GPX proteins. β-actin was used as a loading control.

The ability of cells to up-regulate their intrinsic antioxidant enzymes such as glutathione peroxidase (GPX), catalase, and superoxide dismutase is of fundamental importance in protecting cells and tissues from oxidative damage. Among the different enzymes, GPX4 is responsible for reducing lipid hydroperoxides and cholesterol ester hydroperoxides within cell membranes.[12] In this study, we have provided direct evidence of GPX4 induction by insulin through the PI3K/Akt

signaling and/or low levels of ROS in MCF-7 breast cancer cells. The role of the ROS and PI3K/Akt signaling in the regulation of GPX4 has been the source of much interest lately. The data demonstrate that insulin-induced GPX4 accumulation is sensitive to PI3K/Akt inhibition with LY-294002. This is keeping with results from other work demonstrating the importance of PI3K/Akt activation in GPX4 regulation following cell stimulation with TNF,[13] PDGF,[6] or VEGF.[14]

Furthermore, other studies have reported the ability of ROS to activate PI3K in various cell types.[15,16] Although we did not define the difference, a positive feedback mechanism seems to be involved in the mutual interaction between the ROS and PI3K in a cell type-specific manner. In this regard, it has to be noted that GPX4 may be a point of convergence ofthe Akt and ROS signalings. However, it is unclear whether the ROS and Akt signalings cross-talk with each other or whether they are alternative pathways, which result in the change of GPX4 level.

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