Effect of Insulin on Cell Cycle Progression in MCF-7 Breast Cancer Cells: Direct and Potentiating influence.

Running title: Insulin and Cell Cycle Progression

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Abstract:

We recently demonstrated that in MCF-7 breast cancer cells, insulin promoted the phosphorylation and activation of geranylgeranyl transferase I (GGTI-I), increased the amounts of geranylgeranylated Rho-A, and potentiated the transactivating activity of lysophosphatidic acid (LPA) (Chappell, Golovchenko, Wall, Stjernholm, Leitner, Goalstone, and Draznin, J Biol Chem 275: 31792-31797, 2000). In the present study, we explored the mechanism of this potentiating effect of insulin on LPA. Insulin (10 nM) potentiated the ability of LPA to stimulate cell cycle progression and DNA synthesis in MCF-7 cells. The potentiating effect of insulin appears to involve increases in the expression of cyclin E and decreases in the expression of the cyclin dependent kinase inhibitor p27Kip1. All potentiating effects of insulin were inhibited in the presence of an inhibitor of GGTase I, GGTI-286 (3μM) or by an expression of a dominant negative mutant of Rho-A. In contrast to its potentiating action, a direct mitogenic effect of insulin in MCF-7 cells involves activation of phosphatidylinositol 3-kinase and increased expression of cyclin D1.

We conclude that the ability of insulin to increase the cellular amounts of geranylgeranylated Rho-A results in potentiation of the LPA effect on cyclin E expression and degradation of p27Kip1 and cell cycle progression in MCF-7 breast cancer cells.
Introduction:

Both hyperinsulinemia and cancer are extremely prevalent pathophysiologic conditions associated with major morbidity and mortality. The implication of insulin in the pathogenesis of cancer has been in the English medical literature since the early 1970’s (1). Epidemiological and case-control studies corroborate animal studies consistently showing an increase in pancreatic, breast, colorectal, liver, endometrial and prostate cancer in hyperinsulinemic individuals (2-7). Recently, hyperinsulinemia has been hypothesized as possibly the unifying etiologic factor in the development of colorectal and breast cancer associated with obesity and diabetes (2,8-10). However, the possible molecular mechanisms of insulin’s effect on cancer development and/or progression remain enigmatic.

Insulin is a major mitogen in normal mammary tissue and breast cancer cells in culture (11-14). Many human breast cancers overexpress the insulin receptor (15,16), and overexpression of these receptors can result in a transformed phenotype in human mammary epithelial cells (17,18). Additionally, breast cancer cells fail to down regulate the insulin receptor in the presence of high dose insulin (19,20). One recent study has demonstrated that women with insulin receptor positive breast cancer have worse prognosis than women with insulin receptor-negative tumors (21) and a recent abstract in the American Society of Clinical Oncology (22) indicated that fasting insulin levels predict disease free survival and overall survival of women with breast cancer who are receiving adjuvant therapy.

We have recently identified a novel aspect of insulin action, its ability to stimulate prenylation of small molecular weight GTPases (23-26), which may explain a higher incidence of certain cancers in patients with hyperinsulinemia. Small molecular weight GTPases of the Ras superfamily play an important role in cell proliferation, differentiation, structural organization,
and vesicular trafficking (27,28). The members of this superfamily, including Ras, Rho, and Rab proteins, are activated by GTP loading in response to guanine nucleotide exchange factors (29). Post-translational modification of these proteins by prenylation appears to be a pre-requisite for their subsequent activation (30,31). Hyperinsulinemia significantly increases the activities of farnesyltransferase (FTase) and geranylgeranyltransferases I and II (GGTase I and II) (23-26). Increased activity of these prenyl transferases leads to increases in the amounts of farnesylated p21Ras and geranylgeranylated Rho-A in various tissues (23-26,32). These increases provide more prenylated p21Ras and Rho-A available for activation by other growth promoting agents, thus augmenting cellular mitogenic responses (24,25,32).

We have recently demonstrated in a MCF-7 breast cancer cell line, that insulin promoted the phosphorylation and activation of GGTase I (25). Increases in the GGTase I activity resulted in significant augmentation of the amounts of geranylgeranylated Rho-A in the insulin treated cells and potentiation of the nuclear effects of lysophosphatidic acid (LPA). In the present experiments, we explore the mechanism of the potentiating effect of insulin on LPA-induced cell cycle progression and DNA synthesis in MCF-7 breast cancer cells.

Materials and Methods:

Tissue culture media was from Meditech, Inc. (Herndon VA). Gentamicin was from Life Technologies, Inc. (Gaithersburg, MD). Fetal calf serum (FCS) was from Gemini Bio-Products, Inc. (Calabasas, CA). Bovine serum albumin (BSA), L-α-Lysophosphatidic Acid (LPA) and other biochemicals were from Sigma (St. Louis, MO). Insulin was from Eli Lilly (Indianapolis, IN). Anti-Cyclin E and Anti-Cyclin D1 mouse monoclonal antibodies and
geranylgeranytransferase inhibitor-286 (GGTI-286) were from Calbiochem (San Diego, CA). Anti-p27 and Anti-Rb mouse monoclonal antibodies, protein G-Plus / Protein A-agarose and immunoprecipitation reagents were from Oncogene Science, Inc. (Cambridge, MA). Bicinchoninic acid (BCA) Protein Assay Kit was from Pierce (Rockford, IL). SDS-PAGE supplies and reagents were from Bio-Rad (Hercules, CA). MCF-7 breast cancer cells were a gift from Dr. Carla L. Van Den Berg (University of Colorado Health Science Center, Denver, CO), and a dominant negative mutant of Rho-A was a gift from Dr. J.J. Baldassare (St. Louis University, St. Louis, MO), and BrdU Cell Proliferation ELISA kit was from Boehringer Mannheim (Indianapolis, IN).

**Cell Cycle Analysis**

MCF-7 cells were grown to 80% confluence at 37°C, 5% CO2 in Improved Minimal Essential Media (IMEM) + 5% heat inactivated Fetal bovine serum (FBS), non-essential amino acids, L-glutamine (200 mM) and insulin (60 pM). The cells were serum- and insulin-starved for 24 hours and then pre-incubated with insulin (10 nM) for 24 hours with and without 3 µM GGTI-286, 100 nM wortmannin, or 20 µM PD98059. Cells were then incubated for an additional 24 hours with LPA (20 µM). Cells were lifted from the plates using 2 mL Versene 1:5000 (Gibco/BRL, Gaithersburg, MD), pelleted in 1X phosphate buffered saline and stained with propidium iodide using the method of Krishan (33). Cell cycle analysis was performed using a Coulter Epics XL flow cytometer (Beckman-Coulter, Hialeah, Florida). Alignment of the instrument was verified daily using DNA Check beads (Coulter, Hialeah, Florida). Peak vs. integral gating was used to exclude doublet events from the analysis. Data were collected for 10,000 events. Modfit LT (Verity Software House, Topsham, Maine) was used for cell cycle
modeling.

5-bromo-2′-deoxyuridine (BrdU) Incorporation

MCF-7 cells were grown to 80% confluence in 96 well plates as described above. The cells were serum- and insulin-starved for 24 hours and then pre-incubated with insulin (10 nM) for 24 hours with and without 3 µM GGTI-286. Cells were then incubated for an additional 8 hours with LPA (20 µM) in the presence of 10 µl of BrdU (diluted 1:100 in serum-free medium). Labeling medium was removed and BrdU incorporation was determined by Cell Proliferation ELISA, visualized, and quantified by colorimetry.

Cyclin E and Cyclin D1 Analysis

MCF-7 cells were grown to 80% confluence as described above. The cells were serum- and insulin-starved for 24 hours and then pre-incubated with insulin (10 nM) for 24 hours with and without 3 µM GGTI-286. Cells were then incubated for an additional 24 hours with LPA (20 µM). Cells were washed with PBS and lysed using a Triton X-100 based lysis buffer (50 mM HEPES, pH 7.5, 15 mM MgCl₂, 1 mM PMFS, 1 mM Na₂HPO₄, 1% Triton X-100, 1 mM DTT, 1 mM Na Vanadate, 0.05% SDS, 10 µg/ml Aprotinin, 10 µg/ml Leupeptin), sonicated, centrifuged, and protein concentrations diluted to 1 mg/mL. Cyclin E and Cyclin D₁ protein were immunoprecipitated using the respective antibodies. Immunoprecipitates were resolved by polyacrylamide gel electrophoresis (PAGE), determined by Western blot and quantitated by densitometry.

P27Kip1 and Rb protein Analysis

MCF-7 cells were grown to 80% confluence as described above. The cells were serum- and insulin-starved for 24 hours and then pre-incubated with insulin (10 nM) for 24 hours with and
without 3 µM GGTL-286. Cells were then incubated for an additional 24 hours with LPA (20 µM). Cells were washed with PBS and lysed as above, sonicated, centrifuged, and protein concentrations diluted to 1 mg/mL. P27Kip1 and retinoblastoma (Rb) protein were immunoprecipitated using the respective antibodies. Immunoprecipitates were resolved by PAGE, determined by Western blot and quantitated by densitometry. For the assessment of Rb phosphorylation, the Western blot of the Rb immunoprecipitates was performed using anti-phosphoserine and anti-phosphothreonine antibody.

Statistical analysis:

Statistics were analyzed by Student’s paired or unpaired t-test, with P < 0.05 considered significant.

Results:

Because our recently published observations (35) indicated that hyperinsulinemia potentiates the nuclear effects of LPA by increasing the availability of geranylgeranylated Rho-A, the current experiments were performed to further elucidate the mechanism of the potentiating influence of insulin on growth of MCF-7 cells.

Insulin potentiates the effect of LPA on cell cycle.

In the initial experiments, we examined the direct effects of insulin on MCF-7 breast cancer cell proliferation by fluorescence activated cell cycle analysis. Insulin (10 nM) enhanced cell growth by approximately 40% over control when supplemented in serum free medium (Fig.1). The effect of insulin was dose dependent, with 100 nM insulin enhancing growth by 70% (not shown). Addition of wortmannin (100 nM), an inhibitor of phosphotidylinositol 3-
kinase (PI3-kinase), completely blocked the mitogenic effect of insulin, whereas the MEK inhibitor PD98059 (20µM) had no significant effect on insulin-induced growth (Fig. 1). Thus, these experiments confirmed that the direct mitogenic action of insulin in MCF-7 cells is dependent upon the PI3-kinase branch of insulin's intracellular signaling (34,35).

Because the effect of insulin on GGTase I and geranylgeranylation of Rho-A is mediated by the Shc-Ras-MAP kinase pathway, and is completely independent of PI3-kinase signaling (36), these cells serve as an excellent model to examine the mechanism of the potentiating influence of insulin, independent of its direct mitogenic action which is PI-3-kinase dependent.

We then examined the potentiating effect of insulin on the mitogenic action of LPA. Insulin (10 nM) alone showed the same 40% increase in growth over control, whereas LPA (20 µM) enhanced MCF-7 cell growth 5% over control (Fig. 2). When LPA was added to MCF-7 cells, which were pre-incubated with 10 nM insulin for 24 hours, growth was enhanced to 65% over control. This potentiating effect of insulin was blocked by the addition of the geranylgeranyl transferase inhibitor GGTI-286 (3µM). GGTI-286 had no effect on insulin alone (data not shown). While addition of wortmannin completely blocked the effect of insulin (Fig. 1), it did not affect the ability of insulin to potentiate LPA action to approximately 25% above control vs 5% with LPA alone (Fig. 2). These findings indicate that the potentiating effect of insulin is not dependent on PI 3-kinase. Finally, addition of GGTI to insulin, LPA, and wortmannin completely blocked the potentiating effect of insulin.

Insulin potentiates the effect of LPA on DNA synthesis and cell growth

BrdU incorporation into DNA is another marker of DNA synthesis and cell proliferation. Therefore, we examined the ability of insulin to potentiate the effect of LPA on BrdU
incorporation in MCF-7 cells. Insulin alone increased BrdU incorporation into DNA 18% above control (Fig. 3), whereas LPA alone increased BrdU incorporation by 7%. When LPA was added to cells, which were pre-incubated with 10 nM insulin, BrdU incorporation into DNA was enhanced to 27%. If insulin potentiates the effect of LPA by increasing the availability of prenylated Rho-A, then inhibition of geranylgeranylation of Rho-A should block the potentiating effect of insulin. Indeed, addition of GGTI-286 to the pre-incubation medium blocked the potentiating effect of insulin, significantly decreasing the incorporation of BrdU as compared with the cells incubated with insulin and LPA. BrdU incorporation in cells incubated with insulin in combination with 3 µM GGTI-286 was not significantly different from that observed in experiments with insulin alone (not shown).

In order to confirm the role of Rho-A in mediating the potentiating effect of insulin, cells were transiently transfected with a dominant negative mutant of Rho-A prior to the insulin and LPA challenges. Expression of a dominant negative Rho-A completely blocked the ability of insulin to potentiate the effect of LPA on BrdU incorporation (Fig. 3), without any effect on insulin alone. Because flow cytometry and BrdU incorporation represent surrogate measures of mitogenesis, we performed direct cell count of MCF-7 cells exposed to insulin and LPA (Fig 3B). We observed a synergistic effect of insulin and LPA on cell growth that was completely abolished in the presence of either a GGTase I inhibitor or a dominant negative mutant of Rho-A. Taken together, these experiments suggest that the potentiating effect of insulin is related to the ability of insulin to activate GGTase I, while its direct effect is independent of its action on prenylation.

To assess whether an introduction of a dominant negative Rho-A could squelch the signal transduction proteins, we examined the effect of insulin on the phosphorylation of ERK
1/2 and Akt in control and transfected MCF-7 cells. Transfection of a dominant negative Rho-A had no effect on the ability of insulin to stimulate the phosphorylation of ERK 1/2 and Akt (not shown), suggesting normal functioning of signaling pathways in these cells.

**Insulin potentiates the effect of LPA on cyclin E but not cyclin D production**

Because insulin increased the progression of MCF-7 cells into cell cycle and potentiated the mitogenic effect of LPA, we examined the effect of 10 nM insulin on cell cycle initiation by evaluating its effect on cyclin D1 and cyclin E expression. The aim of these experiments was to identify the point in the initiation of the cell cycle that is potentiated by insulin.

Insulin (10 nM) stimulated cyclin D1 production approximately 30% over control, whereas LPA (20 µM) alone did not stimulate cyclin D1 production over control (Fig. 4A). However, insulin did not potentiate the effect of LPA on cyclin D1. In cells pre-incubated with 10 nM insulin and then exposed to 20 µM LPA, cyclin D1 production was not significantly different from that seen in the cells incubated with LPA alone.

In contrast to the experiments with cyclin D1, insulin (10 nM) alone showed no significant stimulatory effect on cyclin E production, whereas LPA (20 µM) stimulated cyclin E production almost 2 fold (Fig. 4B). However, pre-incubation of MCF-7 cells with 10 nM insulin significantly increased the LPA-stimulated cyclin E production to more than 3 fold as compared with controls. This potentiating effect of insulin was completely abolished with the addition of 3 µM GGTI-286, or by a transient transfection of a dominant negative mutant of Rho-A (Fig. 4B), again suggesting that the potentiating influence of insulin is mediated by its effect on GGTase I.
Insulin potentiates the effect of LPA on Cyclin Dependent Kinase Inhibitors

Because the function of cyclin E is in part regulated by its interactions with the Cdk inhibitor p27\textsuperscript{Kip1} (reviewed in 37,38) and because Rho-A stimulates p27\textsuperscript{Kip1} degradation through its regulation of cyclin E/Cdk 2 activity (39), we examined insulin’s effect on p27\textsuperscript{Kip1} expression in MCF-7 cells. Either insulin (10 nM) or LPA (20 \textmu M) alone showed a mild and non-significant inhibitory effect on p27\textsuperscript{Kip1} production. However, when LPA (20 \textmu M) was added to MCF-7 cells pre-incubated with 10 nM insulin for 24 hours, p27\textsuperscript{Kip1} production was significantly decreased by 33\% (p<0.04) (Fig. 5). The presence of GGTI-286 or transfection of a dominant negative Rho-A completely abrogated a decrease in p27\textsuperscript{Kip1} that was caused by insulin in combination with LPA (Fig. 5).

Regulation of the Rb protein function occurs predominantly through the phosphorylation of serine and threonine residues in the Rb protein predominantly by cyclin D\textsubscript{1}/Cdk 2/6 (reviewed in 40). Phosphorylation of these residues results in the dissociation of the Rb protein from the E2F transcription factor thereby resulting in increased RNA transcription and progression of cells through the restriction point into the cell cycle. We therefore examined insulin’s effect on the phosphorylation of the Rb protein. Although insulin and LPA both increased Rb phosphorylation, we did not observe a potentiating effect of insulin on LPA-mediated Rb phosphorylation (not shown).

Discussion:

Epidemiological studies show that the incidence of breast cancer is increased in women
with hyperinsulinemic disorders including Type 2 diabetes and polycystic ovary syndrome (2-4,10,41), though only slightly greater than in the general population (overall rr~1.6). Most of these epidemiological studies, however, have been based on populations of patients from diabetic clinics without regard to type of diabetes (Type 1 or Type 2). Data from several animal studies show a protective effect from cancer in Type 1 diabetes with a lower incidence of breast cancer (42-44) and tumor regression (43-46) in rats with mammary tumors made diabetic (insulinopenic) using streptozotocin or alloxan. Alternatively, administration of insulin to rats bearing mammary tumors resulted in a significant increase in tumor growth (42). These findings suggest that the epidemiological studies may have underestimated the risk of breast cancer in the hyperinsulinemic state (Type 2 diabetes).

In previous experiments, we have shown insulin’s ability to induce prenylation of low molecular weight GTPases, specifically, farnesylation of Ras protein and geranylgeranylation of Rho-A protein (23-26). These increases in the pool of prenylated small GTPases result in augmentation of DNA synthesis in response to other growth factors and augmentation of Rho-A-mediated transcriptional activation of SRE-dependent genes by LPA (25) as well as transactivation of nuclear factor kB by angiotensin II, hyperglycemia and advanced glycosylation end products (32).

The salient feature of the present investigation is that insulin, in addition to its direct mitogenic effects through the PI3-kinase signaling cascade, potentiates the effect of LPA-mediated MCF-7 cell transition into cell cycle via the Rho-A-dependent activation of cyclin E. Cells pre-incubated with insulin, and then challenged with LPA, exhibited a 65% increase in transition into cell cycle, which was greater than insulin alone (40%), LPA alone (5%), and greater than an additive effect of these two agents. Inhibition of a direct mitogenic effect of
insulin with wortmannin did not affect the ability of insulin to potentiate the effects of LPA. The addition of the geranylgeranyl transferase inhibitor GGTI-286 completely abolished insulin’s potentiating effect on LPA-mediated transition into cell cycle, both in the absence and in the presence of wortmannin. Our experiments with insulin effect on BrdU incorporation into DNA, as a marker for DNA synthesis and cellular growth, mirrored our results form cell cycle analysis. In addition, transfection of a dominant negative mutant of Rho-A also completely prevented the potentiating effect of insulin. Neither GGTI nor the dominant negative Rho-A had any appreciable effect on insulin alone, whereas wortmannin inhibited the direct effect of insulin.

The subsequent set of experiments was designed to identify the point of cell cycle initiation at which insulin exerts its potentiating effect. Because overexpression of either cyclin D1 or cyclin E has been shown to shorten the G1 interval in various mammalian cell lines (47-53), we decided to evaluate the effect of insulin on these early events in G1/S-phase transition. Although insulin increased cyclin D1 expression, it had no potentiating influence on the effect of LPA. In contrast, insulin potentiated the effect of LPA on cyclin E expression, even though it had no influence of its own (Fig. 4A and B). Because the expression of cyclin E is Rho-A-dependent (39,54,55) and because the effects of LPA are mediated via the Rho-A pathway, our results support the hypothesis that the potentiating influence of insulin on LPA is the result of the insulin-induced increases in the availability of prenylated Rho-A (25). Also in support of this explanation is the fact that an inhibitor of GGTase I, GGTI-286, and the dominant negative Rho-A completely prevented the potentiating influence of insulin. In concert with our previous findings that GGTI-286 inhibits insulin’s ability to potentiate the transcriptional action of LPA (25), we now show that GGTI-286 completely inhibits the potentiating effect of insulin on LPA.
actions on cell cycle progression, DNA synthesis, and cyclin E expression.

Cell cycle progression and Cdk function are regulated by complex signaling interactions, which include cyclin dependent kinase (Cdk) inhibitors. Cyclin D-Cdk 4/6 complexes are essential for G1-phase progression and directly bind (56) and phosphorylate the Rb protein (57). In contrast, cyclin E appears to function in both an Rb-dependent and an Rb-independent manner (57). The Cdk inhibitor p27Kip1, on the other hand, inhibits a broader spectrum of Cdks, including cyclin E/Cdk2 (58) and has been shown to decrease dependence of cell proliferation on serum mitogens (59,60). In addition, geranylgeranylated Rho GTPases are essential for the degradation of p27Kip1 and facilitate cell cycle progression (39,61). We therefore examined the effect of insulin and LPA on p27Kip1 expression and Rb phosphorylation. Consistent with the demonstrated influence of insulin on cyclin E, we found that insulin potentiated LPA-mediated down regulation of p27Kip1 protein by 33% (p<0.04).

The present data confirm that insulin per se has a significant mitogenic effect in MCF-7 cells. The major intracellular signaling pathway by which insulin enhances growth of these cells appears to be the PI 3-kinase pathway. The stimulatory effect of insulin on cyclin D expression is also consistent with stimulation of PI 3-kinase pathway as the latter has been shown to control post-translationally the expression of cyclin D (62). In contrast to its direct mitogenic effect, insulin also potentiates the effect of LPA, a major serum mitogen. This potentiating influence of insulin appears to involve the prenylation of Rho-A, expression of cyclin E, and inhibition of p27Kip1. These data are in agreement with our previous findings that insulin activates the prenyltransferases via the Shc-MAP kinase signaling pathway (36,63).
We have previously shown that insulin potentiates EGF- and IGF-1-mediated p21Ras-GTP loading (64), EGF, IGF-1 and PDGF effects on DNA synthesis (24) in 3T3-L1 fibroblasts through increases in the amounts of farnesylated p21Ras. Moreover, insulin potentiates PDGF-mediated VEGF gene expression and thymidine incorporation in vascular smooth muscle cells as well (65). This effect of insulin was also mediated by the increased amounts of farnesylated p21Ras (65). Here, we show that insulin potentiates LPA-mediated cell cycle progression and BrdU incorporation in MCF-7 breast cancer cells and that the mechanism of the enhanced cell cycle progression is through the Rho-A dependent up-regulation of cyclin E and down regulation of the Cdk inhibitor p27Kip1. Because either the presence of GGTI-286 (a geranylgeranyl transferase inhibitor) or the expression of a dominant negative Rho-A completely block the potentiating effect of insulin on all aspects of LPA-mediated cell cycle progression, we postulate that insulin potentiates LPA action through its effect on Rho-A prenylation and provision of increased amounts of geranylgeranylated Rho-A. We have previously shown that the inhibitory effects of GGTI-286 on insulin’s ability to potentiate nuclear effects of LPA are specific and not toxic (25). This is also confirmed by the current data showing no inhibitory effect of GGTI-286 on insulin alone.

Taken together, these data demonstrate that insulin has a significant effect on MCF-7 breast cancer cell growth both in its own right via the PI 3-kinase dependent pathway and through potentiation of LPA-mediated effects by up regulation of prenylation of Rho-A. These data help clarify one of the possible mechanisms by which patients with hyperinsulinemia may have an increased incidence of breast cancer (2-4,10,41). This aspect of insulin action may also explain why women with insulin receptor positive breast cancer have been shown to have a worse
prognosis than women with insulin receptor-negative tumors (21), and why fasting insulin levels may predict disease free survival and overall survival of women with breast cancer who are receiving adjuvant therapy (22).

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**Figure Legends:**

**Figure 1.** Insulin induces MCF-7 cell proliferation through the PI 3-kinase pathway.

MCF-7 cells were incubated with insulin (10nM) with or without wortmannin (WORT, 100 nM) or PD 98059 (20 µM) for 24 hours. Percent of cells in S+G2/M was measured by flow cytometry. Results of 4 experiments are expressed as a percent above control and shown as the mean ± SEM. *, P<0.0001 vs control alone. **, P < 0.01 vs insulin alone

**Figure 2.** Insulin potentiates LPA-mediated MCF-7 cell proliferation.

MCF-7 cells were incubated with or without insulin (INS, 10nM), wortmannin (WORT, 100 nM), and GGTI-286 (GGTI, 3µM) for 24 hours. LPA (20 µM) was added and the cells were then incubated for an additional 24 hours. The percent
of cells in S+G2/M was measured by flow cytometry. Change in the number of cells in S+G2/M is expressed as a percent above control. In this study approximately 28% of control cells were in S+G2/M. Results of 7 experiments are shown as the mean ± SEM. * P<0.0001 vs 10 nM insulin alone; ** P<0.0004 vs 10 nM insulin + 20 µM LPA; # P<0.01 vs LPA alone; ## P, 0.01 vs Ins + LPA + WORT.

Figure 3. Insulin potentiates LPA-mediated BrdU incorporation and cell growth in MCF-7 cells.

Control MCF-7 cells and cells transfected with a dominant negative Rho-A (DNRho) and were incubated with or without insulin (10nM) and GGTI-286 (3µM) for 24 hours. (Panel A) LPA (20 µM) was added and the cells were then incubated for an additional 8 hours in the presence of BrdU. Results of 6 independent experiments (6 plates per group per experiment) are shown as the mean ± SEM of percent incorporation above control. * P<0.001 vs 10 nM insulin alone; # P<0.001 vs Insulin + LPA. (Panel B) Absolute cell count at the end of incubations with designated agents (n = 4). Results are expressed as the mean ± SEM of percent above control. * P < 0.01 vs LPA alone; **, P < 0.001 vs Ins + LPA; #, P < 0.03 vs Ins + LPA.

Figure 4. Insulin potentiates LPA-mediated cyclin E, but not cyclin D1 production.

Control MCF-7 cells and cells transfected with a dominant negative Rho-A
(DNRho) were incubated with or without insulin (10nM) and GGTI-286 (3µM) for 24 hours. LPA (20 µM) was added and the cells were then incubated for an additional 24 hours. Amounts of cyclin D₁ (panel A) and cyclin E (panel B) were determined as described in methods. Results of 10 experiments are shown as the mean ± SEM. * P<0.01 vs LPA alone; ** P<0.001 vs Insulin + LPA.

Figure 5. **Insulin potentiates LPA-mediated downregulation of p27Kip1.**

Control MCF-7 cells and cells transfected with a dominant negative Rho-A (DNRho) were incubated with or without insulin (10nM) and GGTI-286 (GGTI, 3µM) for 24 hours. LPA (20 µM) was added and the cells were then incubated for an additional 24 hours. Results of 7 independent experiments are expressed as the mean ± SEM. * P<0.04 vs control; ** P< 0.05 vs Insulin + LPA.

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Fig. 1

% cells in S+G2/M over control

Control  Insulin  Ins + Wort  PD98059
Fig 4B

Cyclin E

52 kd

Control, Insulin, LPA, Insulin+LPA, Ins+LPA+GGTL, Ins+LPA+DNRIo

Cyclin E

Arbitrary densitometric units

Control, Insulin, LPA, Insulin+LPA, Ins+LPA+GGTL, Ins+LPA+DNRIo
Fig 5

p27Kip

Control  Insulin  LPA  Insulin+LPA  Ins+LPA+GGTI  Ins+LPA+DNRho

*p < 0.05  **p < 0.01

Arbitrary densitometric units

p27 Kip

Control  Insulin  LPA  Insulin+LPA  Ins+LPA+GGTI  Ins+LPA+DNRho

*p < 0.05  **p < 0.01
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