Research article

Insulin utilizes the PI 3-kinase pathway to inhibit SP-A gene expression in lung epithelial cells

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Abstract

Background: It has been proposed that high insulin levels may cause delayed lung development in the fetuses of diabetic mothers. A key event in lung development is the production of adequate amounts of pulmonary surfactant. Insulin inhibits the expression of surfactant protein A (SP-A), the major surfactant-associated protein, in lung epithelial cells. In the present study, we investigated the signal transduction pathways involved in insulin inhibition of SP-A gene expression.
Methods: H441 cells, a human lung adenocarcinoma cell line, or human fetal lung explants were incubated with or without insulin. Transcription run-on assays were used to determine SP-A gene transcription rates. Northern blot analysis was used to examine the effect of various signal transduction inhibitors on SP-A gene expression. Immunoblot analysis was used to evaluate the levels and phosphorylation states of signal transduction protein kinases.
Results: Insulin decreased SP-A gene transcription in human lung epithelial cells within 1 hour. Insulin did not affect p44/42 mitogen-activated protein kinase (MAPK) phosphorylation and the insulin inhibition of SP-A mRNA levels was not affected by PD98059, an inhibitor of the p44/42 MAPK pathway. In contrast, insulin increased p70 S6 kinase Thr389 phosphorylation within 15 minutes. Wortmannin or LY294002, both inhibitors of phosphatidylinositol 3-kinase (PI 3-kinase), or rapamycin, an inhibitor of the activation of p70 S6 kinase, a downstream effector in the PI 3-kinase pathway, abolished or attenuated the insulin-induced inhibition of SP-A mRNA levels.
Conclusion: Insulin inhibition of SP-A gene expression in lung epithelial cells probably occurs via the rapamycin-sensitive PI 3-kinase signaling pathway.

Keywords: insulin, lung epithelial cells, MAPK, PI 3-kinase, surfactant protein A

Introduction

Fetuses of diabetic mothers with uncontrolled blood glucose levels tend to be hyperglycemic and hyperinsulinemic [1]. An increased incidence of neonatal respiratory distress syndrome (RDS) has been observed in infants of diabetic mothers [1]. RDS is caused by inadequate amounts of pulmonary surfactant due to delayed lung development [2]. It

has been proposed that high insulin levels can delay lung development in the fetus of the diabetic mother [3].

Surfactant, a lipoprotein comprised of phospholipids (~80%), cholesterol (~10%), and proteins (~10%), functions to reduce surface tension and prevents alveolar collapse at end expiration [4]. The surfactant-associated proteins (SP) A, B, C and D, which are required for proper

surfactant function, are developmentally and hormonally regulated [4]. We, and others, have shown that the SP-A levels in the amniotic fluid of diabetic mothers are significantly decreased [1]. Low SP-A levels in amniotic fluid have been correlated with an increased incidence of neonatal RDS [5]. Our previous studies have shown that insulin down-regulates SP-A mRNA and protein levels in human lung epithelial cells in a concentration and time-dependent manner via an inhibition of gene transcription [6,7].

Insulin transduces its cellular signal in most cell types via two signaling pathways, i.e., the p44/42 mitogen-activated protein kinase (MAPK) pathway and the phosphatidylinositol 3-kinase (PI 3-kinase) pathway [8]. Insulin initially binds to the insulin receptor causing autophosphorylation of the β-subunit of the receptor [8]. The protein kinase domain of the β-subunit then phosphorylates tyrosine residues in insulin receptor substrate (IRS)-1, IRS-2, IRS-3, or Shc, which then activates the p44/42 MAPK signaling cascade [8]. PD98059 is a cell permeable, non-competitive, reversible inhibitor of MAPK/extracellular signal-regulated kinase kinase (MEK), a protein kinase upstream of p44/42 MAPK [9]. Tyrosine phosphorylated IRS also activates the PI 3-kinase pathway [8,10]. There are at least three, well-characterized inhibitors of the PI 3-kinase pathway, i.e., wortmannin, LY294002, and rapamycin [11–13]. Wortmannin and LY294002 block the activation of PI 3-kinase itself [11,12], while rapamycin prevents the phosphorylation of a downstream effector in the pathway, p70 S6 kinase [13].

In the present study, we used two experimental models, a human lung adenocarcinoma cell line, NCI-H441, and human fetal lung explants. H441 cells resemble bronchiolar epithelial Clara cells in phenotype and produce both SP-A and SP-B mRNA and protein [14]. The hormonal regulation of SP-A and SP-B gene expression in H441 cells is similar to that observed in differentiated type II cells in human fetal lung explants [6,7,15,16]. We treated H441 cells with the four inhibitors of signal transduction: wortmannin, LY294002, rapamycin and PD98059, to elucidate which signaling pathways are activated when insulin inhibits SP-A gene expression. To confirm these results, we repeated key experiments with insulin and the signal transduction inhibitors using human fetal lung explants. We then examined the levels and phosphorylation state of key enzymes in the p44/42 MAPK and PI 3-kinase signaling pathways. We found that insulin probably inhibits SP-A gene expression in lung epithelial cells via the rapamycin-sensitive PI 3-kinase pathway.

Materials and methods
Cell and explant cultures
A human lung adenocarcinoma cell line, NCI-H441, was maintained *in vitro* in monolayer culture in 10% fetal bovine serum in the presence of penicillin (100 U/ml), streptomycin (100 μg/ml) and fungizone (0.25 μg/ml) at 37°C in a 5% CO_2 atmosphere [14]. Culture media were changed every 3 days and cells were passed (1:4) weekly. For the inhibitor experiments, the H441 cells were grown until ~80% confluent, then incubated in serum-free media for 24 hours prior to an experiment. The cultured cells were subsequently exposed to fresh serum-free media and pretreated for 30–60 minutes with either control media that contained the vehicle for the inhibitors (dimethyl sulphoxide) or with media that contained wortmannin (5–200 nM), LY294002 (2–50 μM), rapamycin (1–100 nM), or PD98059 (2.5–25 μM). After pretreatment, either insulin (2.5 μg/ml) or vehicle (dilute HCl) was added for an additional 12–16 hours. Experiments were repeated three to five times unless otherwise noted.

Human lung tissue was obtained from mid-tremester abortuses (15–21 weeks old), dissected free from blood vessels and conducting airways, then minced with a sterile razor blade into ~1 mm^3 explants [6]. The explants were maintained on lens paper-covered stainless steel grids at the air-media interface in Waymouth's media at 37°C and 5% CO_2 for 6 days. The media were changed daily. On the last day of culture, explants were pretreated with signal transduction inhibitors, either wortmannin, rapamycin or PD98059, for 30–60 min and then further treated with insulin (2.5 μg/ml) for 12–24 hours. Experiments were performed in duplicate and were repeated twice.

Reagents
Porcine insulin was purchased from Calbiochem (San Diego, CA, USA), wortmannin, LY294002, and rapamycin were purchased from Sigma-Aldrich Company (St. Louis, MO, USA), and PD98059 was purchased from New England Biolabs (Beverly, MA, USA). Insulin was prepared as 2.5 mg/ml stock solution in ~0.01 N HCl, aliquoted and stored at -80°C. Wortmannin, LY294002, rapamycin and PD98059 were reconstituted in dimethyl sulphoxide as 1 mM, 50 mM, 50 μM and 10 mM stock solutions, respectively, and stored at -80°C in aliquots. Insulin causes a time- and dose-dependent inhibition of SP-A gene expression with maximum effect at 0.25–2.5 μg/ml (~40 to 400 nM) [7]. In order to achieve a maximal inhibitory effect of insulin, we used a concentration of 2.5 μg/ml in the present study.

Transcription run-on assay
Nuclear isolation, transcription elongation reactions and hybridizations were performed as described previously with minor modifications [7]. Subconfluent H441 cells were incubated in serum-free media for 24 hours and then exposed to media plus either vehicle or insulin (2.5 μg/ml) for an additional 1, 4, 8 and 24 hours. The cells were then rinsed and trypsinized, and nuclei from control and treated cells were harvested. The transcription elongation reaction

was performed with 20×10^6 nuclei. Labeled, newly synthesized RNA was then isolated and purified from the nuclei. Nytran membranes with immobilized cDNAs for the BlueScript vector, human SP-A and human α-actin were prehybridized in 1 ml of buffer (50% formamide, 5 X SSC, 5 X Denhardt's solution, 100 μg/ml denatured herring sperm, 0.1% SDS) for 4 hours at 45°C and then hybridized to the labeled RNAs (6×10^6 cpm in the presence of 500 units of RNasin) in duplicate for an additional 60 hours. Afterwards, the hybridized membranes were washed twice in 0.2 X SSC with 0.1% SDS at 55°C for 1 hour, once in 2 X SSC at 55°C for 15 min, once in 2 X SSC with 10 μg/ml RNase A at 37°C for 30 min and then rinsed twice in 2 X SSC at room temperature for 15 min. Membranes were exposed to a Storage Phosphor Screen (Molecular Dynamics, San Francisco, CA, USA) for 3–5 days, scanned using a PhosphorImager (Molecular Dynamics) and data quantitated using Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA).

Northern blot analysis
Northern blot analysis was used to semi-quantitate SP-A mRNA levels. H441 cells were harvested and total RNA isolated as described previously [16]. Equal amounts of total RNA from each condition were separated on agarose gels, transferred to Nytran-plus membranes (Schleicher and Schuell, Keene, NH, USA), and subsequently hybridized with a radiolabeled human SP-A cDNA. Radioactive bands were detected using X-ray film after a 1–4 hour exposure with an intensifier screen at -70°C. The relative intensity of the reactive bands was estimated by densitometry and corrected for RNA loading as described previously [16].

To perform a northern blot analysis of the human fetal lung explant samples, 4 μg of total RNA from each condition were immobilized on Nytran-plus membranes (0.2 μm, Schleicher and Schuell) in triplicate in denaturing solution (66 % formamide, 7.9 % formaldehyde, 26 mM MOPS buffer, pH 7.0, 1.3 mM EDTA, 0.066 M sodium acetate) using a slot blot apparatus. The membranes were then processed for northern blot analysis as described above. In addition, 10 μg of total RNA from each explant sample were separated on agarose gels to ensure the quality of the RNA samples and to correct for RNA loading.

Immunoblotting
The total amount and phosphorylation states of p44/42 MAPK and p70 S6 kinase were assessed using immunoblot analysis according to the methods described by Sharma and coworkers [17]. H441 cells were incubated in serum-free media for 24 hours then exposed to fresh control media plus either insulin (2.5 μg/ml) or vehicle for 15 min, 30 min, 2 hours or 16 hours. Control and treated cells were rinsed twice with PBS and then incubated with 1 ml of lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1.5 mM $MgCl_2$, 50 mM NaF, 5 mM sodium pyrophosphate, 0.2 mM sodium orthovanadate, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 10% glycerol, 1% Triton X-100, 0.5% NP-40, 1 mM phenylmethylsulfonyl fluoride) for 1 hour with shaking at 4°C. Equal amounts of protein lysates from each condition in 1X sample buffer (125 mM Tris-HCl, pH 6.8, 1% β-mercaptoethanol, 2% SDS, 5% glycerol, 0.003% bromphenol blue) were separated on 7.5 or 10% polyacrylamide gels and subsequently transferred to nitrocellulose membranes. Immunoblotting of membranes for phospho-MAPK (extracellular signal-regulated kinase [Erk]1/2) was performed using a Phospho-MAPK Antibody Sampler kit (Cell Signaling, Beverly, MA, USA) according to the manufacturer's instructions. The phospho-MAPK antibody recognizes dual phosphorylated Erk1 and Erk2 at Thr202/Tyr204. Total p44/42 MAPK was detected with rabbit polyclonal anti-MAPK (Erk1) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), according to the manufacturer's instructions. Membranes were then incubated with a secondary antibody, horseradish peroxidase-conjugated anti-rabbit IgG (1:4000, ICN Biomedical Research Products, Costa Mesa, CA, USA). The reactive bands were detected using chemiluminescence (ECL, Amersham Pharmacia Biotech, Piscataway, NJ, USA). Immunoblotting experiments for the detection of total and phosphorylated p70 S6 kinase were performed using a PhosphoPlus p70 S6 Kinase (Thr389, Thr421/Ser424) Antibody kit according to the manufacturer's instructions (Cell Signaling).

Lactate dehydrogenase assay
Lactate dehydrogenase (LDH) activity was determined in cell culture media. Pyruvate conversion into lactate was measured in 0.2 M Tris-HCl (pH 7.2) buffer in the presence of sodium pyruvate (0.037 mg/ml, Fisher Scientific, Fair Lawn, NJ, USA) and the reduced form of β-nicotinamide adenine dinucleotide (0.057 mg/ml, Sigma) at 22°C by measuring absorbance at 340 nm. LDH activity was determined as the rate of decrease in absorbency per min using a standard curve for lactate hydrogenase (2–40 mU, L-lactic dehydrogenase, porcine muscle, Sigma). LDH activity in the media samples was normalized to controls, which were made equal to one.

Statistical analysis
All experiments were repeated at least three times unless otherwise stated. Student's t-test or one-way analysis of variance (ANOVA) followed by Dunnett's test was used to estimate the statistical significance of the results [18].

Results
Lactate dehydrogenase assay
LDH activity was measured in media obtained from control H441 cells and cells treated with the various signal trans-

duction inhibitors. LDH activity was not different from controls in cells treated for 12 to 16 hours with either 200 nM wortmannin, 5 μM LY294002, 20 nM rapamycin, or 10 μM PD98059 (data not shown).

Effects of signal transduction inhibitors and insulin on SP-A mRNA levels

Wortmannin is a fungal metabolite that is a relatively selective inhibitor of PI 3-kinase with a half-maximal inhibition (IC$_{50}$) less than 5 nM [11]. In the wortmannin experiments, media were changed every 2 hours for a total of 12 hours in all of the conditions because wortmannin is unstable in solution at physiological pH [19]. The shorter incubation time with insulin in the wortmannin experiments resulted in a decreased inhibitory effect of insulin, i.e., an ~22% inhibition of SP-A mRNA levels (Fig. 1A) versus an ~40% inhibition observed after the 16-hour incubation used in the experiments with rapamycin and PD98059 (Fig. 1B and 1C). Wortmannin, added alone, did not significantly alter SP-A mRNA levels at any concentration tested (Fig. 1A). However wortmannin, at concentrations of 5–200 nM, abolished the insulin-mediated inhibition of SP-A mRNA levels at all concentrations tested (5–200 nM, Fig. 1A).

To confirm the inhibitory effect of wortmannin on insulin action, a structurally different PI 3-kinase inhibitor, LY294002, was used. LY294002 is a competitive inhibitor of the PI 3-kinase ATP-binding site, with an IC$_{50}$ = 1.4 μM *in vitro*[12]. LY294002, added alone, decreased SP-A mRNA levels at concentrations greater than 10 μM (data not shown). However, 2 μM LY294002 abolished the inhibitory effects of insulin, but did not affect basal SP-A mRNA levels. In the control group, insulin significantly decreased SP-A mRNA levels to 57 ± 11% of control levels, however, in presence of 2 μM LY294002, insulin only decreased SP-A mRNA levels to 87 ± 15% of control levels versus 99 ± 3% of control levels in samples treated with LY294002 alone (mean ± SE, n = 4, n indicates the number of independent experiments).

Rapamycin inhibits activation of p70 S6 kinase, a downstream effector in the PI 3-kinase signaling pathway [13]. Rapamycin forms a complex with FK506-binding protein 12 and this complex blocks the kinase activity of mTOR (mammalian target of rapamycin), a kinase upstream of p70 S6 kinase [20]. The IC$_{50}$ for p70 S6 kinase inhibition by rapamycin is ~0.04–0.4 nM [13]. When added alone at concentrations of 1–100 nM, rapamycin did not affect SP-A mRNA levels (Fig. 1B). However, rapamycin abolished the insulin-mediated inhibition of SP-A gene expression in a dose-dependent manner.

To examine whether the p44/42 MAPK pathway mediates insulin signaling in the H441 cells, PD98059, an inhibitor of the p44/42 MAPK pathway, was used [9]. PD98059

binds to the dephosphorylated form of MEK and prevents its activation *in vivo* with an IC$_{50}$ of 10 μM [9]. PD98059, added alone, significantly decreased SP-A mRNA levels in a dose-dependent manner (Fig. 1C). PD98059 had no effect on the insulin inhibition of SP-A mRNA levels.

We also treated cultured human fetal lung explants with wortmannin, rapamycin or PD98059 in the presence or absence of insulin. Fig. 2 shows that wortmannin (100 nM) and rapamycin (50 nM) both abolished the insulin-mediated inhibition of SP-A gene expression. In agreement with the H441 cells results, PD98059 (2.5–10 μM) had no effect on the insulin inhibition of SP-A mRNA while decreasing basal SP-A mRNA levels in a dose-dependent manner when added alone.

Transcription rate of human SP-A

In our previous studies, we have shown that insulin inhibits the rate of SP-A gene transcription after a 24-hour exposure [7]. In this study, we evaluated the effects of insulin on the transcription rate of the human SP-A and α-actin genes at several time-points earlier than those used in our previous studies. The rate of SP-A gene transcription increased in a time-dependent manner (Fig. 3). Insulin inhibited human SP-A gene transcription at every time-point tested, even as early as after 1 hour of insulin treatment. Insulin did not decrease human α-actin gene transcription at any time point.

Phosphorylation-activation of protein kinases

Immunoblot analysis of control and insulin-treated H441 cells using phosphospecific antibodies for p44/42 MAPK and p70 S6 kinase revealed that both kinases were in a partially activated state at the 15 min time point. The phosphorylation of the protein kinases in the controls declined over time and remained at minimum levels after 30 min to 2 hours of incubation. We attribute this partial activation of the signaling kinases to the media change that occurred immediately prior to insulin or vehicle addition [21].

p44/42 MAPK

The total amount of p44/42 MAPK was equal and remained level in control and insulin-treated cells at all time-points (Fig. 4A). Insulin did not affect p44/42 MAPK phosphorylation within a 2-hour incubation time, but significantly decreased phosphorylation after 16 hours (Fig. 4B). Densitometric data of immunoreactive bands of phospho-p44/42 MAPK in the presence of insulin were equal to 0.39 ± 0.01 for the 16 hour time point when compared to the corresponding controls which were made equal to one (mean ± SE, t-test, p < 0.05, n = 3).

Since it is possible that the insulin-mediated stimulation of p44/42 MAPK phosphorylation had ended within a 15 min exposure to insulin, we performed additional experiments in

Figure 1
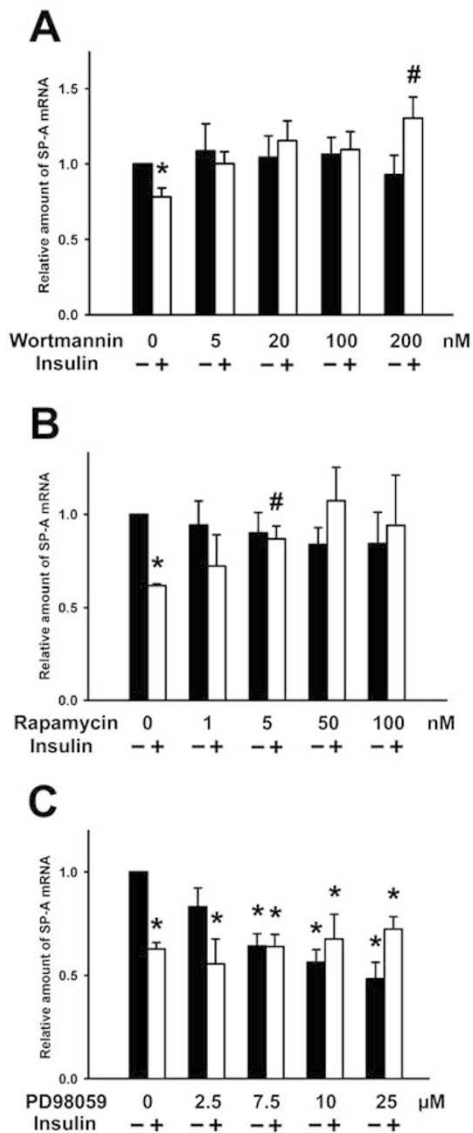
SP-A mRNA levels in the presence or absence of insulin (2.5 μg/ml) and/or signal transduction inhibitors in H441 cells. SP-A mRNA bands were analyzed by densitometry and levels in control cells were made equal to one. Data are presented as the mean ± SE (standard error of the mean). Asterisks indicate a significant difference when compared to the control condition (ANOVA, Dunnett's test, p < 0.05 or t-test, p < 0.05), # indicates a significant difference from the insulin alone condition (ANOVA, Dunnett's test, p < 0.05), n indicates the number of independent experiments. (A) Wortmannin, an inhibitor of PI 3-kinase (5–200 nM), did not affect SP-A mRNA levels at any concentration when added alone. Wortmannin blocked the inhibitory effect of insulin on SP-A mRNA at every concentration (n = 4). (B) Rapamycin, an inhibitor of p70 S6 kinase activation, added alone (1–100 nM), did not affect SP-A steady-state mRNA levels. Rapamycin blocked the inhibitory effects of insulin on SP-A gene expression in a dose-dependent manner (n = 3). (C) PD98059, an inhibitor of p44/42 MAPK (2.5–25 μM), inhibited SP-A mRNA levels in dose-dependent manner. At 2.5 μM, a concentration that did not affect basal SP-A mRNA levels, PD98059 did not reverse the inhibitory effect of insulin (n = 5). ANOVA = analysis of variance; MAPK = mitogen-activated protein kinase; SP = surfactant protein

order to evaluate the possibility of a shorter time course of insulin action. Serum-starved cells were incubated with fresh serum-free media for 1 hour at 37°C (in order to return the phosphorylation levels of p44/42 MAPK to the basal state) and then treated with insulin (2.5 μg/ml) or vehicle for 5 min. Insulin did not activate p44/42 MAPK within this time frame. The intensity of the immunoreactive bands of phospho-p44/42 MAPK from insulin-treated cells was 0.92 ± 0.10 relative to controls, which were made equal to one (data are the mean of two experiments performed in duplicate plus or minus the standard deviation of the mean). In order to prove that p44/42 MAPK can be stimulated in H441 cells by an activator of this kinase, some cells were also treated with 10 nM 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA), a strong stimulator of p44/42 MAPK in H441 cells, for 5 min [21]. TPA caused an approximately twofold increase in p44/42 MAPK phosphorylation over control levels.

p70 S6 kinase
The total amount of p70 S6 kinase present in the cells decreased during the 16 hour incubation period, however, there was no difference in the amount present in control vs. insulin-treated cells at any time-point (Fig. 5A). Fig. 5B shows that despite increased basal p70 S6 kinase phosphorylation, insulin caused an additional up-regulation of p70 S6 kinase on Thr389 after incubation with insulin for 15 min to 16 hours. Phosphorylation of p70 S6 kinase on Thr389 is essential for the activation of the kinase [22]. In the presence of insulin, the intensity of the Thr389 p70 S6 kinase bands was significantly increased after 15 min exposure and reached 2.02 ± 0.31 after 15 min and 1.36 ± 0.17 after a 16 hour incubation period when compared to respective controls which were made equal to one (mean ± SE, t-test, p < 0.05, n = 4). Insulin also stimulated the phosphorylation of p70 S6 kinase Thr421/Ser424 at the 30-min and 2-hour incubation times when compared to their respective controls (Fig. 5C).

Inhibitors of p44/42 MAPK and p70 S6 kinase
PD98059 caused a dramatic decrease in the phosphorylation of p44/42 MAPK, in the presence or absence of insulin (Fig. 6A) when compared to controls. Incubation of cells with rapamycin did not affect p44/42 MAPK phosphorylation in any condition (Fig. 6B).

Rapamycin completely abolished the phosphorylation of Thr389 in p70 S6 kinase in insulin-treated cells and also abolished the basal phosphorylation of p70 S6 kinase in control cells, at both time points examined (Fig. 6C). Rapamycin also down regulated the Thr421/Ser424 phosphorylation in p70 S6 kinase in control and insulin-treated cells at both time-points (Fig. 6E). PD98059 did not affect Thr389 or Thr421/Ser424 phosphorylation in p70 S6 kinase in insulin-treated cells at either time point, but slightly

Figure 2
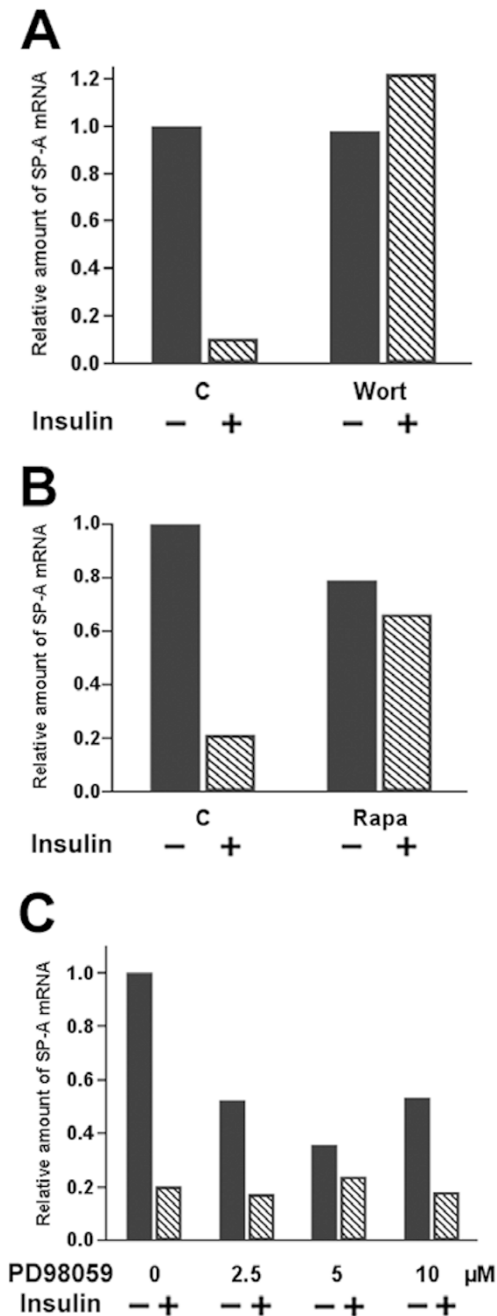
SP-A mRNA levels in the presence or absence of insulin (2.5 µg/ml) and/or signal transduction inhibitors in human fetal lung explants. Explants were grown for 6 days, then treated with inhibitors plus or minus insulin or vehicle for 12 hours (wortmannin experiments) or 24 hours (rapamycin and PD98059 experiments), harvested and analyzed for human SP-A mRNA. Data representative of two experiments with similar results are shown. (A) Wortmannin (100 nM) abolished the insulin-mediated inhibition of SP-A gene expression. (B) Rapamycin (50 nM) inhibited the insulin-mediated decrease in SP-A mRNA level. (C) PD98059 (2.5–10 µM) decreased basal SP-A mRNA levels in a dose-dependent manner, but had no effect on the insulin-mediated inhibition of SP-A gene expression. SP = surfactant protein

Figure 3
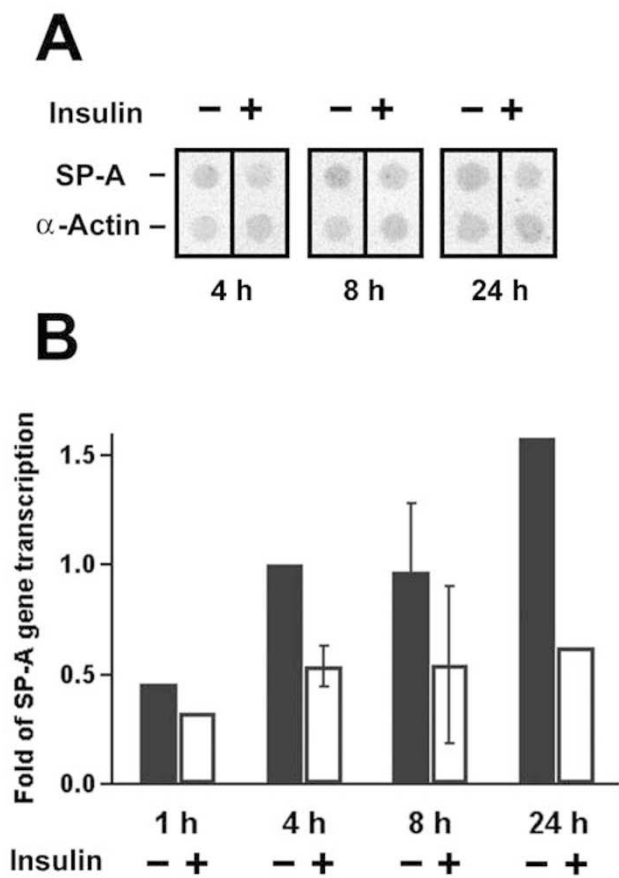
Transcription of the human SP-A genes in the presence or absence of insulin (2.5 µg/ml). The level of transcription in control cells at the 4-hour time-point was made equal to one. The data are the results of three experiments expressed as the mean ± SD (standard deviation of the mean). (A) A representative phospho-image of human SP-A and human α-actin gene transcription in H441 cells treated with or without insulin for the time periods indicated. (B) Insulin inhibited human SP-A gene transcription as early as after 1 hour of insulin exposure, with a maximum effect observed after 24 hours when compared to controls. SP = surfactant protein

decreased their phosphorylation when added alone (Figs. 6D and 6F).

Discussion
Nothing is known about the relationship between signaling pathways and the regulation of SP-A gene expression by insulin. In contrast, insulin signal transduction pathways are well-characterized in a wide variety of cell types [8,10]. Insulin is known to primarily transduce its cellular actions via two signaling pathways, the p44/42 MAPK pathway and the PI 3-kinase pathway [8,10]. In the present study, we investigated the signaling pathways that mediate the inhibitory effects of insulin on the expression of the SP-A genes in human lung epithelial cells.

Figure 4
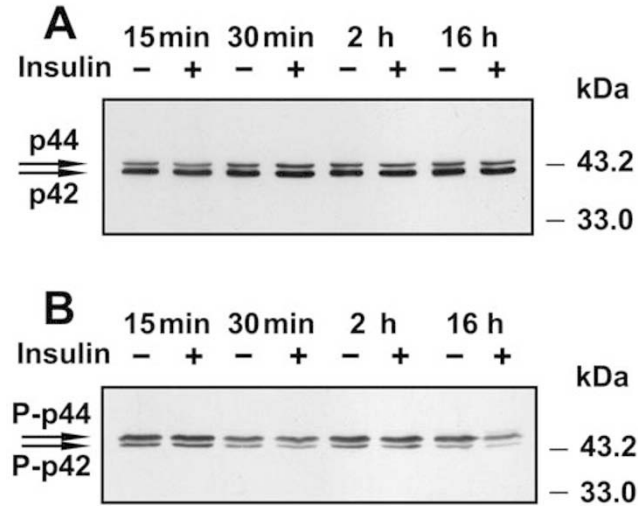
p44/42 MAPK in H441 cells treated with or without insulin (2.5 µg/ml). Equal amounts of protein homogenate from each condition were separated by electrophoresis then probed by immunoblotting. Protein molecular weight standards (kDa) are shown on the right. The data are representative of 3 experiments. (A) Total MAPK (p44 and p42, arrows). The total amount of MAPK was not altered by insulin treatment at any time-point. (B) Phospho-p44 and p42 MAPK (P-p44 and P-p42, arrows). Insulin had no effect on the phosphorylation of p44/42 MAPK when the cells were incubated with insulin for 15 min to 2 hours. After a 16-hour incubation, insulin decreased the levels of phospho-p44/42 MAPK. MAPK = mitogen-activated protein kinase

Two inhibitors of PI 3-kinase, wortmannin and LY294002, and an inhibitor of p70 S6 kinase activation, rapamycin, blocked the inhibitory effect of insulin on SP-A gene expression. We confirmed these observations by showing that insulin increased the phosphorylation of a specific rapamycin-sensitive residue (threonine 389), present in the linker region of p70 S6 kinase, a phosphorylation event that is known to activate this enzyme [22]. In contrast, PD98059, an inhibitor of p44/42 MAPK activation, did not block the inhibitory effects of insulin on SP-A gene expression. In addition, insulin had no effect on p44/42 MAPK phosphorylation during a 2-hour incubation. The effects of the PI 3-kinase and the MAPK pathway inhibitors on SP-A gene expression were evaluated using two experimental models, a human immortalized epithelial cell line (H441 cells) and human fetal lung explants. We also demonstrated that transcriptional inhibition of SP-A gene commences within 1 hour of exposure to the hormone. Together, our results are suggestive that insulin activates the rapamycin-sensitive PI 3-kinase pathway in lung epithelial cells and that this event leads to an inhibition of SP-A gene transcription.

We found that wortmannin and rapamycin had no effect on basal SP-A mRNA levels, at any concentration tested. However, PD98059, added alone, abolished p44/42

Figure 5
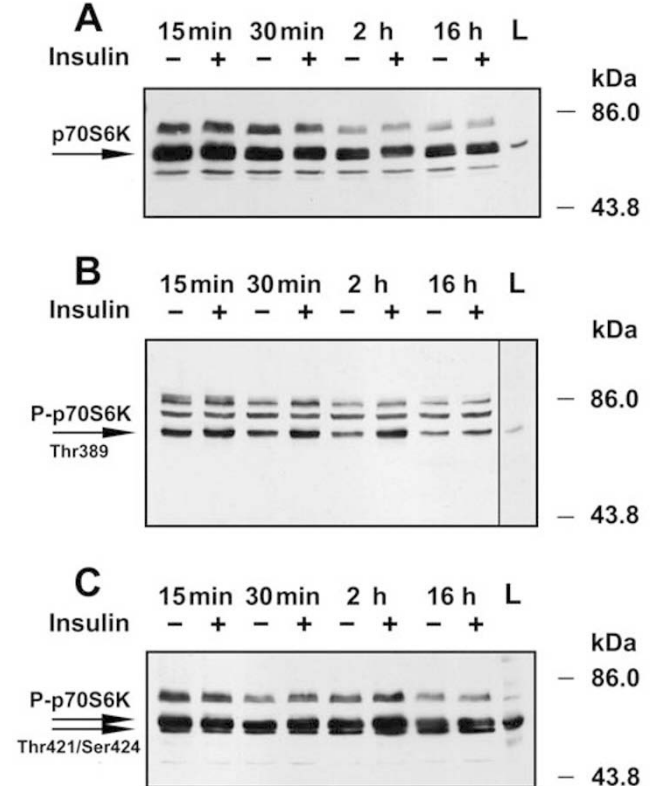
p70 S6 kinase in H441 cells in the presence or absence of insulin (2.5 µg/ml). Equal amounts of total protein were separated by gel electrophoresis and then probed by immunoblot analysis. Protein molecular weight standards (kDa) are shown on the right. A total lysate of NIH-3T3 cells treated with serum (L) served as a positive control for p70 S6K detection. The data are representative of four experiments. (A) Detection of total p70 S6 kinase. The total amount of p70 S6 kinase declined over time in both the control and insulin-treated conditions with the least amount of protein detected after a 16-hour incubation. There was no effect of insulin on the total amount of p70 S6 kinase when compared to respective control cells at any time point. (B) Detection of phosphorylated p70 S6 kinase. Insulin increased the phosphorylation of Thr389 in p70 S6 kinase at every time point. (C) Detection of phospho-p70 S6 kinase. Insulin increased the phosphorylation of Thr421/Ser424 in p70 S6 kinase after a 30 min and 2 hour incubation time when compared to controls.

MAPK phosphorylation in control cells and also inhibited basal SP-A mRNA levels in a dose-dependent manner. We hypothesize that a basal level of p44/42 MAPK activity may be required for SP-A gene expression. Chess and coworkers have shown that transforming growth factor-α and hepatocyte growth factor increase the phosphorylation of p44/p42 MAPK in H441 cells [23]. Epidermal growth factor, which binds to the same receptor as transforming growth factor-α, increases SP-A content in human fetal lung [24]. Hepatocyte growth factor has also been shown to upregulate the synthesis of SP-A in rat type II cells [25]. Thus, phosphorylation of p44/42 MAPK may be involved in the up-regulation of SP-A gene expression, results consist-

Figure 6
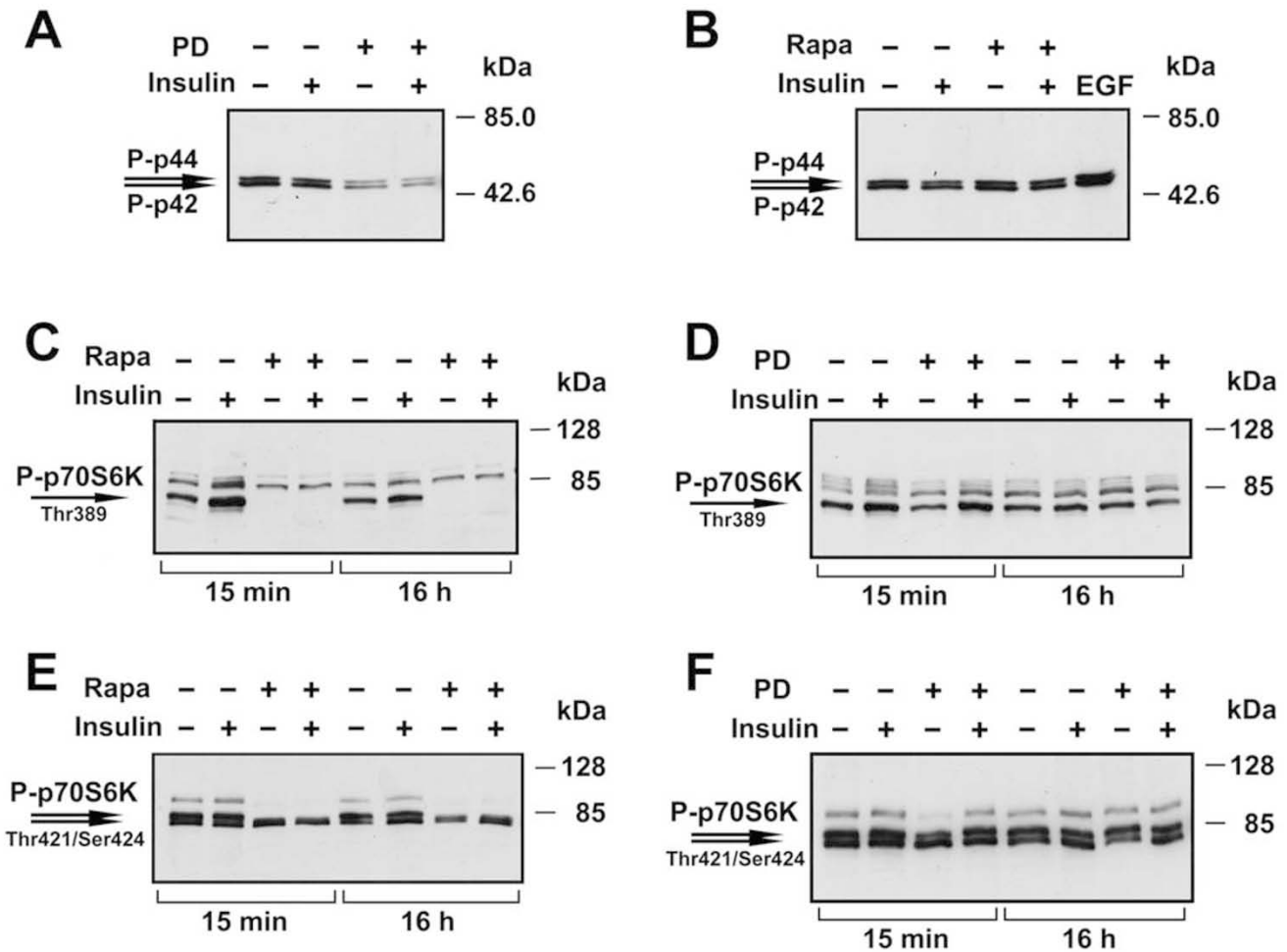
p44/42 MAPK and p70 S6 kinase in cells incubated in the presence of insulin (2.5 µg/ml), PD98059 (10 µM) and/or rapamycin (50 nM). Cells were harvested and immunoblotted for phosphorylated p44/42 MAPK and p70 S6 kinase, then the immunoblots were stripped and reprobed for total MAPK and p70 S6 kinase. The data are representative of two experiments. Protein molecular weight standards (kDa) are shown on the right. (A) When the cells were pretreated with PD98059, the phosphorylation of p44/42 MAPK was greatly decreased in both control and insulin-treated cells after 16 hours. (B) Rapamycin did not affect the phosphorylation of p44/42 MAPK after 16 hours. (C) The phosphorylation of Thr389 in p70 S6 kinase was increased in the presence of insulin after 15 min of incubation and remained increased after 16 hours. Rapamycin abolished the phosphorylation of Thr389 in both control and insulin-treated cells at the 15 min and 16-hour incubation times. (D) Insulin increased the phosphorylation of Thr389 p70 S6 kinase after 15 min and 16-hour incubations. PD98059 did not affect the insulin-induced up-regulation at 15 min. (E) Rapamycin decreased the phosphorylation of Thr421/Ser424 in p70 S6 kinase after 15 min and 16 hours in control and insulin-treated cells. (F) PD98059 added alone caused a slight decrease in phosphorylation of Thr421/Ser424 p70 S6 kinase in control cells treated for 15 min and 16 hours but did not affect the phosphorylation in insulin-treated cells. MAPK = mitogen-activated protein kinase

ent with our observation that inhibition of basal levels of p44/42 MAPK phosphorylation may decrease SP-A gene expression.

In summary, our studies are suggestive that in lung epithelial cells, SP-A gene transcription is inhibited by insulin via the rapamycin-sensitive PI 3-kinase pathway, but not the p44/42 MAPK pathway. In cultured human muscle cells, only the PI 3-kinase – p70 S6 kinase pathway, and not the MAPK pathway, mediates insulin up-regulation of p85α PI

3-kinase gene expression [26]. Similarly, in L6 myotubes, insulin induces hexokinase II gene transcription via the rapamycin-sensitive PI 3-kinase pathway, but not via the MAPK pathway [27]. The PI 3-kinase – p70 S6 kinase pathway, but not the MAPK pathway, mediates insulin induction of glucose-6-phosphate dehydrogenase gene expression in primary rat hepatocytes [28]. In H411E liver cells, insulin down-regulates cAMP-induced phosphoenolpyruvate carboxykinase gene transcription via the PI 3-kinase pathway, but not via the MAPK pathway [29]. Phos-

phorylated p70 S6 kinase has been shown to be present in the nucleus in insulin-treated cells [30]. Moreover, it has been shown that p70 S6 kinase phosphorylates and activates members of the cAMP response element modulator family of transcription factors [31]. These data raise the possibility that transcriptional regulation of SP-A gene expression occurs via the PI 3-kinase – p70 S6 kinase pathway. Further studies will be required to elucidate the function of p70 S6 kinase in the nuclear events involved in SP-A gene transcription.

Conclusion

Our studies are suggestive that insulin inhibits SP-A gene transcription via the PI 3-kinase pathway in lung epithelial cells, specifically via the rapamycin-sensitive activation of p70 S6 kinase. Insulin does not activate p44/42 MAPK and does not inhibit SP-A gene transcription via the MAPK pathway. Our findings further characterize the signal transduction pathways that control surfactant protein gene expression. This information could lead to the possible modification of these pathways by pharmacological agents in order to modulate the composition of pulmonary surfactant, which is critical for normal respiratory function and for host defense mechanisms in the lung.

Abbreviations

ANOVA = analysis of variance; Erk= extracellular signal-regulated kinase; IC_{50} = half-maximal inhibition; IRS = insulin receptor substrate; LDH = lactate dehydrogenase; MAPK = mitogen-activated protein kinase; MEK = MAPK/extracellular signal-regulated kinase kinase; mTOR = mammalian target of rapamycin; PBS = phosphate-buffered saline; PI 3-kinase = phosphatidylinositol 3-kinase; RDS = respiratory distress syndrome; SD = standard deviation of the mean; SE = standard error of the mean; SP = surfactant protein; TPA = 12-O-tetradecanoylphorbol-13-acetate.

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References

1. Snyder JM, George TN, Miakotina OL: **Insulin and lung development.** *In Contemporary Endocrinology: Endocrinology of the lung: Development and surfactant synthesis. (Edited by: Mendelson CR) Totowa, NY: Humana Press Inc.* 2000, 181-200
2. Avery ME, Mead J: **Surface properties in relation to aletectasis and hyaline membrane disease.** *Am J Dis Child* 1959, **97:**517-523
3. Robert MF, Neff RK, Hubbell JP, Taeusch HW, Avery ME: **Association between maternal diabetes and the respiratory-distress syndrome in the newborn.** *N Engl J Med* 1976, **294:**357-360
4. Weaver TE, Whitsett JA: **Function and regulation of expression of pulmonary surfactant-associated proteins.** *Biochem J* 1991, **273:**249-264
5. Pryhuber GS, Hull WM, Fink I, McMahan MJ, Whitsett JA: **Ontogeny of surfactant proteins A and B in human amniotic fluid as indices of fetal lung maturity.** *Pediatr Res* 1991, **30:**597-605
6. Dekowski SA, Snyder JM: **Insulin regulation of messenger ribonucleic acid for the surfactant-associated proteins in human fetal lung in vitro.** *Endocrinology* 1992, **131:**669-676
7. Miakotina OL, Dekowski SA, Snyder JM: **Insulin inhibits surfactant protein A and B gene expression in the H441 cell line.** *Biochim Biophys Acta* 1998, **1442:**60-70
8. Myers MG Jr, White MF: **Insulin signal transduction and the IRS proteins.** *Annu Rev Pharmacol Toxicol* 1996, **36:**615-658
9. Dudley DT, Pang L, Decker SJ, Bridges AJ, Saltiel AR: **A synthetic inhibitor of the mitogen-activated protein kinase cascade.** *Proc Natl Acad Sci U S A* 1995, **92:**7686-7689
10. Shepherd PR, Withers DJ, Siddle K: **Phosphoinositide 3-kinase: the key switch mechanism in insulin signalling.** *Biochem J* 1998, **333:**471-490
11. Arcaro A, Wymann MP: **Wortmannin is a potent phosphatidylinositol 3-kinase inhibitor: the role of phosphatidylinositol 3,4,5-trisphosphate in neutrophil responses.** *Biochem J* 1993, **296:**297-301
12. Vlahos CJ, Matter WF, Hui KY, Brown RF: **A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002).** *J Biol Chem* 1994, **269:**5241-5248
13. Chung J, Kuo CJ, Crabtree GR, Blenis J: **Rapamycin-FKBP specifically blocks growth-dependent activation of and signaling by the 70 kd S6 protein kinases.** *Cell* 1992, **69:**1227-1236
14. O'Reilly MA, Gazdar AF, Morris RE, Whitsett JA: **Differential effects of glucocorticoid on expression of surfactant proteins in a human lung adenocarcinoma cell line.** *Biochim Biophys Acta* 1988, **970:**194-204
15. Metzler MD, Snyder JM: **Retinoic acid differentially regulates expression of surfactant-associated proteins in human fetal lung.** *Endocrinology* 1993, **133:**1990-1998
16. George TN, Snyder JM: **Regulation of surfactant protein gene expression by retinoic acid metabolites.** *Pediatr Res* 1997, **41:**692-701
17. Sharma RV, Tan E, Fang S, Gurjar MV, Bhalla RC: **NOS gene transfer inhibits expression of cell cycle regulatory molecules in vascular smooth muscle cells.** *Am J Physiol* 1999, **276:**H1450-H1459
18. Zar J: *Biostatistical Analysis. New York: Prentice-Hall,* 1984
19. Kimura K, Hattori S, Kabuyama Y, Shizawa Y, Takayanagi J, Nakamura S, Toki S, Matsuda Y, Onodera K, Fukui Y: **Neurite outgrowth of PC12 cells is suppressed by wortmannin, a specific inhibitor of phosphatidylinositol 3-kinase.** *J Biol Chem* 1994, **269:**18961-18967
20. Brown EJ, Beal PA, Keith CT, Chen J, Shin TB, Schreiber SL: **Control of p70 s6 kinase by kinase activity of FRAP in vivo.** *Nature* 1995, **377:**441-446
21. Awasthi V, King RJ: **PKC, p42/p44 MAPK, and p38 MAPK are required for HGF-induced proliferation of H441 cells.** *Am J Physiol Lung Cell Mol Physiol* 2000, **279:**L942-L949
22. Pearson RB, Dennis PB, Han JW, Williamson NA, Kozma SC, Wettenhall RE, Thomas G: **The principal target of rapamycin-induced p70s6k inactivation is a novel phosphorylation site within a conserved hydrophobic domain.** *EMBO J* 1995, **14:**5279-5287
23. Chess PR, Ryan RM, Finkelstein JN: **H441 pulmonary epithelial cell mitogenic effects and signaling pathways in response to HGF and TGF-alpha.** *Exp Lung Res* 1998, **24:**27-39
24. Whitsett JA, Weaver TE, Lieberman MA, Clark JC, Daugherty C: **Differential effects of epidermal growth factor and transforming growth factor-beta on synthesis of Mr = 35,000 surfactant-associated protein in fetal lung.** *J Biol Chem* 1987, **262:**7908-7913
25. Xu X, McCormick-Shannon K, Voelker DR, Mason RJ: **KGF increases SP-A and SP-D mRNA levels and secretion in cultured rat alveolar type II cells.** *Am J Respir Cell Mol Biol* 1998, **18:**168-178
26. Roques M, Vidal H: **A phosphatidylinositol 3-Kinase/p70 ribosomal S6 protein kinase pathway is required for the regulation by insulin of the p85alpha regulatory subunit of phosphatidylinositol 3-kinase gene expression in human muscle cells.** *J Biol Chem* 1999, **274:**34005-34010
27. Osawa H, Sutherland C, Robey RB, Printz RL, Granner DK: **Analysis of the signaling pathway involved in the regulation of hexokinase II gene transcription by insulin.** *J Biol Chem* 1996, **271:**16690-16694
28. Wagle A, Jivraj S, Garlock GL, Stapleton SR: **Insulin regulation of glucose-6-phosphate dehydrogenase gene expression is rapamycin-sensitive and requires phosphatidylinositol 3-kinase.** *J Biol Chem* 1998, **273:**14968-14974
29. Agati JM, Yeagley D, Quinn PG: **Assessment of the roles of mitogen-activated protein kinase, phosphatidylinositol 3-kinase, protein kinase B, and protein kinase C in insulin inhibition of**

cAMP-induced phosphoenolpyruvate carboxykinase gene transcription. *J Biol Chem* 1998, **273:**18751-18759

30. Kim SJ, Kahn CR: **Insulin stimulates p70 S6 kinase in the nucleus of cells.** *Biochem Biophys Res Commun* 1997, **234:**681-685

31. de Groot RP, Ballou LM, Sassone-Corsi P: **Positive regulation of the nuclear activator CREM by the mitogen-induced p70 S6 kinase.** *Immunobiology* 1995, **193:**155-160