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Synergy in ERK activation by cytokine receptors and tyrosine kinase growth factor receptors

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Summary

Epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) signal through EGF and PDGF receptors, which are important receptor tyrosine kinases (RTKs). Growth hormone (GH) and prolactin (PRL) are four helical bundle peptide hormones that signal via GHR and PRLR, members of the cytokine receptor superfamily. In this study, we examine crosstalk between signaling pathways emanating from these disparate receptor groups (RTKs and cytokine receptors). We find that GH and EGF specifically synergize for activation of ERK in murine preadipocytes. The locus of this synergy resides at the level of MEK activation, but not above this level (ie., not at the level of EGFR, SHC, or Raf activation). Furthermore, dephosphorylation of the scaffold protein, KSR, at a critical serine residue is also synergistically promoted by GH and EGF, suggesting that GH sensitizes these cells to EGF-induced ERK activation by augmenting the actions of KSR in facilitating MEK-ERK activation. Similarly specific synergy in ERK activation is also detected in human T47D breast cancer cells by cotreatment with PRL and PDGF. This synergy also resides at the level of MEK activation. Consistent with this synergy, PRL and PDGF also synergized for c-fos-dependent transactivation of a luciferase reporter gene in T47D cells, indicating that events downstream of ERK activation reflect this signaling synergy. Important conceptual and physiological implications of these findings are discussed.

1. Introduction

Growth hormone (GH) and prolactin (PRL) are pituitary-derived protein hormones that have powerful physiological and pathophysiological actions. GH strongly induces multiple growth promoting and metabolic effects [1,2] by virtue of its interaction with the cell surface GH receptor (GHR), a single membrane-spanning glycoprotein member of the cytokine receptor superfamily [3]. GHR is expressed in many tissues, most prominently in liver, muscle, and fat, but also in breast and other tissues [4-6]. PRL is structurally similar to GH

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and also has many effects, particularly with regard to breast development and lactation [7,8]. Indeed, both GHR and PRL receptor (PRLR) have been implicated in breast cancer pathogenesis [9-13]. PRLR and GHR are homologous proteins (in humans, 32% extracellular domain identity; less in the intracellular domain) [14]. GHR and PRLR both activate the non-receptor tyrosine kinase, JAK2, and engage intracellular signaling molecules including STAT5 isoforms, the Ras-MEK-ERK pathway, and the phosphatidylinositol 3 kinase pathway, among others [4,15,16].

In addition to signaling via their own receptors, both GH and PRL have complex relationships with other receptors and signaling pathways. For example, GH and PRL have each been shown to interact with the insulin-like growth factor-1 receptor (IGF-1R) and IGF-1 signaling pathways [17-20]. Likewise, both GH and PRL can impact signaling through the epidermal growth factor receptor (EGFR) family of receptors [21-25]. In particular, GH activation of the ERK signaling pathway causes phosphorylation of EGFR at the intracellular residue Thr-669, resulting in modulation of EGF-induced EGFR tyrosine phosphorylation and downregulation. The net effect in certain cell types is that GH and EGF can synergize in terms of the pace and extent of signal transduction that they elicit, at least in part by virtue of GH's ability to potentiate and sustain EGF-induced EGFR signaling [22,23,25]. PRL has similar effects on EGF-induced EGFR signaling and trafficking in human breast cancer cells [24].

In distinction to cytokine receptor superfamily members such as GHR and PRLR, the EGFR is a receptor tyrosine kinase (RTK). RTKs are a diverse collection of cell surface transmembrane proteins that are grouped into multiple subfamilies, based on structural and functional similarities within the subfamilies [26]. All RTKs possess a ligand-binding extracellular domain, a transmembrane domain, and an intracellular domain in which are embedded a tyrosine kinase domain and regulatory regions. EGF is a 53 amino acid peptide that has important roles in cell growth, differentiation, motility and adhesion, by virtue of binding to EGFR and/or its related subfamily members [27]. The platelet-derived growth factor receptor (PDGFR) is an important member of another RTK subfamily [26]. Its ligand, PDGF, has a wide array of important effects on mitogenesis, migration, and development [28]. For both EGFR and PDGFR, ligand binding triggers a number of signaling pathways, including the Ras-Raf-MEK-ERK pathway, which is biologically critical for downstream actions.

In this study, we further pursue signaling synergy between GH and EGF in murine preadipocytes and extend this concept to examine similar relationships between the PRL and platelet-derived growth factor (PDGFR) signaling systems in the setting of human breast cancer cells. We observed that GH and EGF each acutely activated ERK, Akt, and phospholipase C- γ (PLC- γ) in preadipocytes. Combined treatment with GH and EGF did not augment more than additively either Akt or PLC- γ however, ERK was synergistically activated by combined GH and EGF treatment, consistent with our previous findings. Analysis of the ERK pathway indicated that signaling synergy did not occur at the levels of EGFR, SHC, or Raf activation, but combined GH and EGF treatment resulted in MEK activation that exceeded the sum of GH- and EGF-induced MEK activation, suggesting that ERK signaling synergy resides at the level of MEK activation by Raf. Interestingly, this correlated with synergy in dephosphorylation of the adaptor protein, KSR, suggesting that GH potentiates EGF-induced KSR-mediated MEK-ERK activation. These findings of selective synergistic ERK activation were paralleled by our observations that combined treatment of human breast cancer cells with PRL and PDGF yielded synergistic ERK activation and expression of a luciferase reporter driven by a c-fos response element. Collectively, our data suggest that common mechanisms underlie ERK pathway signaling synergy between cytokine receptors and RTKs.

2. Materials and methods

2.1. Materials

Recombinant human GH was kindly provided by Eli Lilly Co. (Indianapolis, IN). Recombinant human EGF was purchased from Upstate Biotechnology (Lake Placid, NY). Recombinant human PDGF-BB was purchased from R&D Systems (Minneapolis, MN). Recombinant human PRL was obtained through the National Hormone and Pituitary Program of NIDDK (Dr. A.F. Parlow, Harbor-UCLA Medical Center, Torrance, CA).

2.2. Antibodies

Polyclonal anti-EGFR, polyclonal anti-PLC- γ , polyclonal anti-phospho-PLC- γ , polyclonal anti-phosphoAKT, polyclonal anti-AKT, monoclonal anti-STAT5 (G2), anti-phosphoRaf-1, anti-Raf-1, anti-MEK1/2, anti-KSR antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), polyclonal anti-phospho-EGFR antibodies anti-pTyr-845, anti-pTyr-992, and anti-pTyr-1068, and anti-pSer-392 KSR antibody (Cell Signaling Technology, Beverly, MA), anti-active mitogen-activated protein kinase affinity-purified rabbit antibody (anti-active ERK, recognizing the dually phosphorylated Thr-183 and Tyr-185 residues corresponding to the active forms of ERK1 and ERK2 (Promega, Madison, WI), anti-mitogen-activated protein kinase affinity-purified rabbit antibody (recognizing both ERK1 and ERK2) (Upstate Biotechnology, Lake Placid, NY), anti-phosphoMEK1/2 antibody (Biosource International, Inc, Camarillo, CA), polyclonal anti-SHC antibody (BD Transduction Laboratories), and polyclonal anti-pSTAT5 (Zymed Laboratories, San Francisco, CA), were all purchased commercially.

2.3. Cell culture

3T3-F442A cells, kindly provided by Drs. H. Green (Harvard University, Boston, MA) and C. Carter-Su (University of Michigan, Ann Arbor, MI), were cultured in Dulbecco's modified Eagle's medium containing 4.5g/liter glucose (Cellgro, Inc.), supplemented with 10% calf serum, 100 units/ml penicillin, 100 μ g/ml streptomycin (all from Biofluids, Rockville, MD). 3T3-L1 cells from American Type Culture Collection (Manassas, VA) were grown in the above medium, supplemented with 10% fetal bovine serum (Biofluids, Rockville, MD) instead of 10% calf serum. HEK-293-Jak2 cells grows in Dulbecco's modified Eagle's medium containing 1.0g/liter glucose (Cellgro, Inc), supplement with 10% fetal bovine serum. The human breast cancer cell line, T47D, was purchased from ATCC (Manassas, VA) and cultured in RPMI 1640 medium (Cellgro Inc., Herndon, VA, USA), supplemented with 10% fetal bovine serum, 50 μ g/ml gentamicin sulfate, 100 U/ml penicillin and 100 μ g/ml streptomycin (all from Biofluids, Rockville, MD, USA).

2.4. Cell starvation, inhibitor pretreatment, cell stimulation, and protein extraction

Serum starvation of 3T3-F442A cells was accomplished by substitution of 0.5% (w/v) bovine serum albumin (fraction V Roche Molecular Biochemicals) for fetal bovine serum in the culture medium for 16-20h prior to experiments. Pretreatments and stimulations were carried out at 37°C in binding buffer (consisting of 25mM Tris-HCL (pH 7.4), 120mM NaCl, 5mM KCl, 1.2mM MgCl₂, 0.1% (w/v) bovine serum albumin, and 1mM dextrose). Stimulations were terminated by washing the cells once with ice-cold phosphate-buffered saline supplemented with 0.4 mM sodium orthovanadate (PBS-vanadate) and then harvested by scraping in PBS-vanadate. Cells were collected by brief centrifugation, and pelleted cells were solubilized for 30 min at 4°C in lysis buffer (1% (v/v) Triton X-100, 150 mM NaCl, 10% (v/v) glycerol, 50 mM Tris-HCl (pH 8.0), 100 mM NaF, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 mM benzamidine, 5 μ g/ml aprotinin, and 5 μ g/ml leupeptin). After centrifugation at 15,000 $\times$ g for 15 min at 4°C, the

detergent extracts (supernatant) were subjected to immunoprecipitation or were directly electrophoresed and immunoblotted, as indicated below.

2.5. Immunoprecipitation and immunoblotting

For immunoprecipitation, cell extracts (500-1000 μ g) were mixed with 5 μ l of polyclonal anti-KSR antibody (1 μ g) and incubated at 4°C overnight with continuous agitation. Protein G-sepharose beads (Amersham Biosciences, Piscataway, NJ) were added and incubated at 4°C for an additional hour. The beads were washed four times with lysis buffer adjusted to 0.5% (v/v) Triton X-100. Laemmli sample buffer eluates were resolved by SDS-PAGE and immunoblotted as indicated below.

Proteins resolved by SDS-PAGE were transferred to Hybond ECL nitrocellulose membranes (Amersham Biosciences). The membranes were blocked with TBST buffer (20mM Tris-HCl (pH 7.6), 150mM NaCl, and 0.1% (v/v) Tween 20) containing 2% (w/v) bovine serum albumin and incubated with primary antibodies (0.5-1 μ g/ml) as specified in each experiment. After three washes with TBST, the membranes were incubated with appropriate secondary antibodies (1:10,000 dilution) and washed. The bound antibodies were detected with SuperSignal chemiluminescent substrate (Pierce Chemical Co., Rockford, IL). Membrane stripping was performed according to the manufacturer's suggestions (Amersham Biosciences).

2.6. c-fos luciferase transactivation assay

T47D cells (75% confluent in a 100 $\times$ 20mm dish) were transfected with the plasmid p2FTL containing a luciferase reporter gene driven by two tandem copies of a c-fos enhancer fragment [29] using LipofectAMINE Plus reagents (Invitrogen, San Diego, CA, USA). The cells were split into one 12-well plate 18-20h after transfection and grown overnight in culture medium containing 10% fetal bovine serum. The cells were serum-starved for 8h and then stimulated with vehicle, PRL or PDGF or combined stimulation, as indicated in figure legends, in triplicate for 16h. Cells were lysed with luciferase lysis buffer (200 μ l/well), and 100 μ l of cell lysates were used for luciferase activity assay as described previously [24,30,31].

2.7. Densitometric analysis

Densitometric quantitation of ECL immunoblots was performed using a high-resolution scanner and the ImageJ 1.30 program (developed by W.S. Rasband, Research Services Branch, National Institute of Mental Health, Bethesda, MD). Pooled data from several experiments are displayed as mean $\pm$ S.E. The significance of differences (*p* value) of pooled results was estimated using paired *t* tests.

3. Results

3.1 GH and EGF synergize in ERK activation in murine preadipocytes

Our work and that of others has shown that murine 3T3-F442A fibroblast preadipocytes are highly responsive to both GH and EGF and can be used to study crosstalk between these two signaling pathways [21,23,31-36]. We first compared acute ERK activation induced by each ligand to that resulting from costimulation. Serum-starved cells were treated with vehicle, GH (500 ng/ml; 40 min), EGF (1 nM; 30 min), or the combination (GH pretreatment for 10 min followed by EGF for 30 min), after which detergent cell extracts were resolved by SDS-PAGE and immunoblotted to detect the levels of ERK and its activated form. A representative immunoblot is shown in Figure 1A and densitometric evaluation of several such experiments is shown in Figure 1B. Consistent with our previous observations [23], the transient GH-induced ERK activation in these cells subsided after 30 min of continuous

exposure. However, EGF-induced ERK activation was more sustained and easily detected. Notably, costimulation resulted in ERK activation which was significantly greater than that detected when the individual responses to GH and EGF were summed. This synergistic increase of EGF-induced ERK activation afforded by GH costimulation was also detected in 3T3-L1 preadipocytes and in HEK-293 cells that transfected to express GHR and JAK2 (data not shown).

Both GH and EGF acutely activate other signaling pathways in common; among these are the signaling enzymes PLC- γ and Akt [4,27,37-39]. Using the same treatment paradigm as for ERK activation, we tested the effects of GH and EGF, individually or in combination, on PLC- γ (Figure 2A,B) and Akt (Figure 2C,D) activation in 3T3-F442A cells. Each ligand caused acute phosphorylation of both PLC- γ and Akt under these conditions. Costimulation with GH and EGF further enhanced both PLC- γ and Akt phosphorylation compared to either ligand alone; however, in contrast to the findings for ERK activation, quantitation revealed a lack of synergy in PLC- γ and Akt activation for GH plus EGF cotreatment compared to the summed individual treatments.

STAT5 is a critical effector of GH-induced JAK2 activation [40-42]. As expected, GH caused robust tyrosine phosphorylation of STAT5 in 3T3-F442A cells (Figure 2E). In contrast, no STAT5 activation was detected in response to EGF stimulation. Combination GH and EGF exposure did not further augment GH-induced STAT5 phosphorylation, a conclusion supported by quantitative densitometric analysis of several such experiments (Figure 2F). Collectively, the data in Figure 2 suggest that GH/EGF signaling synergy in murine preadipocytes is largely ERK pathway-specific.

3.2 GH augmentation of EGF-induced ERK activation in 3T3-F442A cells resides at the Raf-MEK level

Like other growth factors, EGF activates ERKs mainly by engaging the Grb-2-SOS-Ras-Raf-MEK-ERK pathway [43]. EGF binding causes EGFR tyrosine kinase activation and tyrosine autophosphorylation of the receptor cytoplasmic tail. This recruits the small adaptor protein, Grb-2, and its associated guanine nucleotide exchange factor, SOS, to the EGFR at the plasma membrane, allowing activation of membrane-associated Ras and consequent recruitment of the Raf serine kinase. Raf phosphorylates and activates MEK, which thereby phosphorylates and activates ERKs 1 and 2. GH activates the same cascade, but can also use the adaptor proteins SHC, IRS-1, and Gab-1 to access the pathway [44-48]. As our data suggest that GH synergistically augments EGF-induced ERK activation in 3T3-F442A cells, we sought to localize the level(s) in EGF-induced downstream ERK signaling at which GH exerts this effect.

We first examined EGFR tyrosine phosphorylation resulting from acute EGF, GH, or combined GH plus EGF treatment (Figure 3A). We previously showed that treatment of 3T3-F442A cells with GH alone for 15 min caused EGFR phosphorylation detected with antibodies specific for pY845, pY992, and pY1068 [23]. Using our current treatment paradigm, only mild pY992 signal was detected after 25 min of GH exposure. EGF, in contrast, caused robust phosphorylation at each of these three tyrosine residues, as expected. Notably, however, no increased phosphorylation at any of these sites was detected with combination GH plus EGF treatment, suggesting that the source of the GH/EGF synergy in ERK activation was not the result of GH augmentation of EGF-induced EGFR tyrosine phosphorylation or activation. Similarly, we assessed tyrosine phosphorylation of SHC (Figure 3B) and serine phosphorylation of Raf (Figure 3C-E); these modifications are associated with activation of these molecules along the ERK activation pathway. In neither case did GH synergize with EGF, indicating that the ERK activation synergy did not result at either of these levels in the pathway.

In distinction to the findings for SHC and Raf, experiments testing GH, EGF, and combination treatment in 3T3-F442A cells revealed compelling evidence for synergy in MEK activation (Figure 4A,B). Although brief GH treatment induced substantial MEK phosphorylation (not shown), pMEK signal largely subsided after 40 min. EGF treatment for 30 min briskly activated MEK phosphorylation. Notably, GH pretreatment for 10 min followed by EGF for 30 min yielded nearly 40% greater MEK activation than that accounted for by the summed signals of GH alone plus EGF alone. These data strongly suggest that GH's ability to synergistically augment EGF-induced ERK activation resides at the level of Raf's ability to activate MEK. Kinase suppressor of Ras (KSR) is a scaffolding protein that facilitates ERK activation by fostering association of Raf, MEK, and ERK. KSR interacts with 14-3-3 by virtue of constitutive phosphorylation on serine-392 of KSR. Dephosphorylation of this residue by the phosphatase PP2A releases KSR and its associated MEK, which are then translocated to the plasma membrane in proximity of activated Raf, facilitating ERK activation [49]. We monitored KSR serine-392 phosphorylation by immunoblotting with a phosphospecific pS392 antibody (Figure 5A,B). GH treatment itself had no effect on serine-392 phosphorylation, while treatment with EGF alone was associated with modest KSR serine-392 dephosphorylation (~23% reduction compared to untreated cells). However, the combination of EGF and GH reduced KSR serine-392 phosphorylation by roughly 46% compared to control. These compelling results lead us to conclude that GH facilitates the synergistic lessening of KSR serine-392 phosphorylation, suggesting that the mobilization of this important EGFR scaffold is an important part of GH's sensitizing effect on EGF-induced ERK activation.

3.3 PRL specifically augments PDGF-induced MEK/ERK activation in human T47D breast cancer cells

We sought to determine whether selective GH augmentation of EGF-induced ERK activation was mirrored by interaction of another cytokine receptor-engaging hormone (PRL) with another RTK-engaging growth factor (PDGF). For these experiments, we used the estrogen receptor- and progesterone receptor-positive human breast cancer cell line, T47D. We and others have amply demonstrated that T47D responds to PRL with activation of JAK2, ERKs, STAT5, and other pathways [24,50-55]. Likewise, these cells have been shown to be PDGF-responsive [56,57]; however, in T47D there has been less definition of PDGF signaling (in particular, as pertains to the ERK pathway) as compared with PRL. Thus, we first documented PDGF-induced ERK and MEK activation (Figure 6A,B). Serum-starved T47D cells were treated with PDGF-BB (1 ng/ml) for the indicated durations. Detergent solubilized proteins were resolved by SDS-PAGE and immunoblotted to detect pERK (Figure 6A) and pMEK (Figure 6B). Both were acutely and transiently activated in response to PDGF with pMEK peaking at 5-15 min and pERK peaking at 15-30 min. To examine combined effects of PRL and PDGF, a similar protocol was employed as with GH and EGF stimulation of 3T3-F442A cells, except the treatment durations for PRL and PDGF in T47D cells were briefer. PRL (500 ng/ml) pretreatment for 10 min followed by PDGF (1 ng/ml) treatment yielded substantially greater ERK activation than either PRL alone (15 min) or PDGF alone (5 min) or the summation of these individual signals (Figure 6C,D), indicating synergistic ERK activation by combination of the two ligands.

As with GH/EGF cotreatment in 3T3-F442A cells, we also examined the effects of PRL and PDGF on other signal pathways in T47D cells, using the same protocol as that followed for evaluation of ERK activation. In these cells, PDGF had relatively little or no effect on activation of Akt (Figure 7A,B) or STAT5 (Figure 7C,D), pathways that were both activated by PRL treatment. Notably, combination treatment did not further augment either of these signals compared to individual treatment, indicating that PDGF could not in some way

facilitate PRL-induced signaling via these pathways and that the synergy observed for ERK activation was relatively pathway-specific.

As we found in 3T3-F442A for GH/EGF cotreatment, no synergy was observed in T47D cells at the level of Raf activation for PRL/PDGF cotreatment (not shown). However, evaluation of MEK activation yielded a striking synergy (Figure 8A,B). In these experiments, the level of MEK phosphorylation observed during combination PRL/PDGF treatment was nearly 2.5-fold greater than that calculated to be the summed response of individual treatment with PRL plus individual treatment with PDGF. These data for MEK activation are qualitatively quite similar to those obtained for ERK activation in these cells. We conclude that the MEK activation is the main locus of synergy in the ERK activation pathway in both systems.

3.4 PRL synergizes with PDGF for transactivation of a c-fos enhancer-driven reporter gene in human T47D breast cancer cells

To extend our observations on ERK pathway signaling in T47D cells, we assessed the degree to which PRL, PDGF, or combined treatment influenced ERK-dependent gene expression. For these experiments, we utilized a well-characterized reporter gene in which firefly luciferase is driven by two tandem copies of a c-fos enhancer fragment (the p2FTL reporter plasmid) [24,29-31], which is known to be ERK-responsive. Serum-starved cells that were divided from a pool that was transiently transfected with p2FTL were treated in triplicate with PRL, PDGF, or the combination of the two ligands. After 16 h, cell lysates were assayed for luciferase activity and the fold-increase over control treated cells was measured. In the representative experiment shown in Figure 9A, PDGF and PRL each caused increased luciferase activity, the effect of PDGF alone being more modest than that of PRL alone under the experimental conditions. Coincubation, however, produced substantially greater luciferase activity, significantly exceeding the summed responses to the individual treatments. Results from several such experiments were pooled and the observed response to the combined treatment exceeded that of the summed individual treatments by greater than 50%, on average (Figure 9B). These results suggest that the synergistic acute ERK activation observed in these cells in response to PRL plus PDGF is reflected by transactivation of the c-fos-driven luciferase reporter assayed 16 h thereafter.

4. Discussion

In this study, we further explore crosstalk between GH and EGF signaling and extend our findings to peptide hormones (PRL and PDGF) that signal through a cytokine receptor (PRLR) related to the GHR and an RTK (PDGFR) that shares some signaling mechanisms with the EGFR. We find that GH and EGF selectively synergize in terms of ERK pathway activation in murine preadipocytes and that the ability of GH pretreatment to sensitize the cells to EGF for ERK activation resides not at the EGFR, SHC, or Raf activation levels, but rather at the level of MEK-ERK activation. Furthermore, we observe that EGF-induced dephosphorylation of KSR at a site that corresponds to KSR's activation as scaffold that augments MEK-ERK activity is markedly enhanced by GH, suggesting that a substantial component of GH's ability to potentiate acute EGF-induced ERK activation resides in its capacity to activate KSR. In breast cancer cells, we observed similarly selective synergy between PRL and PDGF for ERK activation that also resided at the level of MEK activation. In these cells, we demonstrated PRL-PDGF synergy in transactivation of a c-fos-driven reporter gene, suggesting that the synergistic ERK activation brought about by combination treatment with these peptides resulted in similarly synergistic consequences downstream of ERK.

EGFR is known to interact in several important ways with other signaling systems. Some non-EGF stimuli can transactivate the EGFR by virtue of proteolytically releasing EGF-like ligands from their molecular precursors, thereby increasing their local concentrations and activating EGFR's intrinsic kinase activity and signaling pathways [58-60]. This form of crosstalk with EGFR has not been reported for GH or for PRL. However, GH can cause EGFR tyrosine phosphorylation at several tyrosine residues without activation of EGFR's intrinsic kinase activity [23,32]. This can allow docking of SH2-containing signaling molecules with EGFR and thereby effect GH-dependent signals.

GH (and PRL) can also promote phosphorylation of EGFR on a site(s) other than tyrosine that is recognized by an antibody that detects phosphorylation of ERK substrates [22-25]. In particular, threonine-669 in the EGFR proximal cytoplasmic domain is a target of such GH-induced phosphorylation [25]. The net effect is that brief pretreatment with GH or PRL, via ERK-dependent EGFR phosphorylation, blunts subsequent EGF-induced EGFR downregulation and prolongs EGFR downstream signaling [23-25].

Our current findings add another layer of complexity to the relationship between GHR and EGFR signaling. In addition to GH using EGFR as a docking molecule to facilitate downstream GHR signal propagation and GH modulating EGF-induced EGFR trafficking to potentiate EGFR signaling, our data indicate that GH also sensitizes the EGF-induced EGFR-mediated ERK pathway such that it is more activatable at a post receptor (and, specifically, post-Raf) level. This augmentation appears to reside with GH's ability to activate KSR to more efficiently activate MEK. However, further work will be necessary to identify specific mechanisms by which the GH-EGF synergy observed in 3T3-F442A occurs. Furthermore, our data do not allow us to definitively know which stimulus sensitizes the other, yet the synergy occurs with kinetics most consistent with GH potentiating EGF signaling rather than vice-versa.

This view is supported by the findings related to the KSR scaffold protein. Dephosphorylation of KSR on serine-392 is seen as potentiating ERK activation by releasing sequestered KSR and allowing it to fulfill its scaffolding role [49]. In 3T3-F442A, GH stimulation had no effect under the conditions examined on the level of phosphorylation at this site, while EGF itself significantly reduced this phosphorylation. Cotreatment further augmented KSR dephosphorylation induced by EGF. At this point, we do not know what aspect of GH signaling confers this potentiation of KSR serine-392 dephosphorylation. Understanding of this aspect of synergy will be quite revealing; along these lines, whether GH activates the protein phosphatase, PP2A, will be an important avenue of investigation.

Our observation in T47D cells of synergy in ERK activation between PRL and PDGF signaling is, to our knowledge, the first of its kind to be reported. As with GH-EGF synergy in other cells, future studies will be needed to isolate definitively the molecular underpinnings of this synergy; indeed, the mechanisms of our observed GH-EGF and PRL-PDGF synergies may differ. However, the finding that downstream ERK-dependent gene transactivation by the combination of PRL and PDGF substantially exceeded that of the sum of each individual stimulus may have important pathophysiological consequences, as both PRL and PDGF have been implicated as players in tumorigenesis and cancer behavior.

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Figure 1. GH/EGF synergy for ERK activation in 3T3-F442A preadipocytes

A. Serum-starved 3T3-F442A cells were stimulated with GH (500ng/ml; 40 min), EGF (1nM; 30 min), GH pretreatment for 10 min followed by EGF stimulation for 30 min, or vehicle. Detergent extracts were resolved by SDS-PAGE and immunoblotted with anti-active ERK1/2 and anti-ERK1/2. **B.** Data shown in **A**, along with those obtained from 2 other experiments were subjected to densitometry analysis. Data are normalized to the level of ERK activation induced by cotreatment (ERK activation induced by EGF following GH pretreatment is considered as 100%). Data are mean $\pm$ S.E. of three experiments. The sum of those induced by EGF alone plus GH alone (referred to as GH+EGF) is calculated by summation of ERK activation induced by EGF and GH. Comparison of the level of ERK1/2 activation induced by cotreatment with sum of each individual treatment is indicated.

Figure 2. Lack of GH/EGF synergy for PLC- γ , Akt, and STAT5 activation in 3T3-F442A preadipocytes

A, C, D. Serum-starved 3T3-F442A cells were treated with vehicle, GH, EGF, or GH plus EGF, as in Figure 1. Detergent extracts were resolved by SDS-PAGE and sequentially immunoblotted with anti-pPLC- γ and anti-PLC- γ (A), anti-pAkt and anti-Akt (C) and anti-pSTAT5 and anti-STAT5 (E). B, D, F. Data shown in A, C, and E, along with those obtained from two other experiments for each, were subjected to densitometry analysis. In each experiment, the cotreatment-induced PLC- γ phosphorylation (B), Akt phosphorylation (D), or STAT5 phosphorylation (F) is considered as 100%. Data are mean $\pm$ S.E. of three experiments. Signals observed by cotreatment (G/E) are compared to the sum of those induced by EGF alone plus GH alone (referred to as GH+EGF), as in Figure 1. $p > 0.05$ in all cases.

Figure 3A-C

Figure 3D,E

Figure 3. Lack of GH/EGF synergy for EGFR tyrosine phosphorylation, SHC tyrosine phosphorylation, and Raf activation in 3T3-F442A preadipocytes

A-D. Serum-starved 3T3-F442A cells were treated with vehicle, GH, EGF, or GH plus EGF, as in Figure 1, for the indicated durations. Detergent extracts were resolved by SDS-PAGE and immunoblotted separately with anti-pY1068, anti-pY992, anti-pY845, and anti-EGFR (A), sequentially with anti-pSHC and anti-SHC (B) and sequentially with anti-pRaf and anti-Raf (C,D). E. Data shown in D, along with those obtained from two other experiments, were subjected to densitometry analysis. In each experiment, the cotreatment-induced Raf phosphorylation is considered as 100%. Data are mean $\pm$ S.E. of three experiments. Signals observed by cotreatment (G/E) are compared to the sum of those induced by EGF alone plus GH alone (referred to as GH+EGF), as in Figure 1. $p > 0.05$.

Figure 4. GH/EGF synergy for MEK activation in 3T3-F442A preadipocytes

A. Serum-starved 3T3-F442A cells were treated with vehicle, GH, EGF, or GH plus EGF, as in Figure 1, for the indicated durations. Detergent extracts were resolved by SDS-PAGE and sequentially immunoblotted with anti-pMEK and anti-MEK. **B.** Data shown in A, along with those obtained from two other experiments, were subjected to densitometry analysis. In each experiment, the cotreatment-induced MEK phosphorylation is considered as 100%. Data are mean $\pm$ S.E. of three experiments. Signals observed by cotreatment (G/E) are compared to the sum of those induced by EGF alone plus GH alone (referred to as GH+EGF), as in Figure 1.

Figure 5. GH/EGF synergy for KSR pS392 dephosphorylation in 3T3-F442A preadipocytes
A. Serum-starved 3T3-F442A cells were treated with vehicle, GH, EGF, or GH plus EGF, as in Figure 1, for the indicated durations. Detergent extracts were resolved by SDS-PAGE and sequentially immunoblotted with anti-pS392 KSR and anti-KSR. **B.** Data shown in A, along with those obtained from three other experiments, were subjected to densitometry analysis. In each experiment, the untreated KSR pS392 phosphorylation is considered as 100%. Data are mean $\pm$ S.E. of four experiments. Signals observed by cotreatment (G/E) are compared to the sum of those induced by EGF alone plus GH alone (referred to as GH+EGF), as in Figure 1.

Figure 6. PRL/PDGF synergy for ERK activation in T47D human breast cancer cells

A,B. Kinetics of PDGF-induced MEK/ERK activation. Serum-starved T47D cells were treated with PDGF (1 ng/ml) for the indicated durations. Detergent extracts were resolved by SDS-PAGE and sequentially immunoblotted with anti-pERK1/2 and anti-ERK1/2 (*A*) and anti-pMEK and anti-MEK (*B*). Data shown are representative of three separate experiments. *C.* Serum-starved T47D cells were stimulated with PRL (500ng/ml; 15 min), PDGF (1 ng/ml; 5 min), PRL pretreatment for 10 min followed by PDGF stimulation for 5 min, or vehicle. Detergent extracts were resolved by SDS-PAGE and immunoblotted with anti-active ERK1/2 and anti-ERK1/2. *D.* Data shown in *C*, along with those obtained from 3 other experiments were subjected to densitometry analysis. Data are normalized to the level of ERK activation induced by cotreatment (ERK activation induced by PDGF following PRL pretreatment is considered as 100%). Data are mean±S.E. of four experiments. The sum of those induced by PDGF alone plus PRL alone (referred to as PRL+PDGF) is calculated by summation of ERK activation induced by PDGF and PRL. Comparison of the level of ERK1/2 activation induced by cotreatment with the sum of each individual treatment is indicated.

Figure 7. Lack of PRL/PDGF synergy for Akt and STAT5 activation in T47D cells

A, C. Serum-starved T47D cells were treated with vehicle, PRL, PDGF, or PRL plus PDGF, as in Figure 6. Detergent extracts were resolved by SDS-PAGE and sequentially immunoblotted with anti-pAkt and anti-Akt (*A*) and anti-pSTAT5 and anti-STAT5 (*C*). *B, D.* Data shown in *A, C*, along with those obtained from three or four other experiments for each, respectively, were subjected to densitometry analysis. In each experiment, the maximum treatment-induced Akt phosphorylation (*B*), or STAT5 phosphorylation (*D*) is considered as 100% in each experiment. Data are mean $\pm$ S.E. of three (*B*) or four (*D*) experiments. Signals observed by cotreatment (P/P) are compared to the sum of those induced by PDGF alone plus PRL alone (referred to as PRL+PDGF), as in Figure 6. $p > 0.05$ in all cases.

Figure 8. PRL/PDGF synergy for MEK activation in T47D cells

A. Serum-starved T47D cells were stimulated with PRL (500ng/ml; 15 min), PDGF (1 ng/ml; 5 min), PRL pretreatment for 10 min followed by PDGF stimulation for 5 min, or vehicle. Detergent extracts were resolved by SDS-PAGE and immunoblotted with anti-pMEK and anti-MEK. **B.** Data shown in A, along with those obtained from 3 other experiments were subjected to densitometry analysis. Data are normalized to the level of MEK activation induced by cotreatment (MEK activation induced by PDGF following PRL pretreatment is considered as 100%). Data are mean±S.E. of four experiments. The sum of those induced by PDGF alone plus PRL alone (referred to as PRL+PDGF) is calculated by summation of MEK activation induced by PDGF and PRL. Comparison of the level of MEK activation induced by cotreatment with the sum of each individual treatment is indicated.

Figure 9. PRL and PDGF synergistically transactivate a *c-fos*-luciferase reporter gene

A. T47D cells were transfected with the p2FTL plasmid (a luciferase reporter gene driven by two tandem copies of a *c-fos* enhancer fragment). Serum-starved cells were then stimulated with vehicle, PRL (500ng/ml), PDGF (1ng/ml), or costimulated with PRL and PDGF in triplicate for 16h, after which luciferase activity was measured. Fold increase is indicated for each condition with the basal luciferase activity (treated with vehicle) set as 1.

Costimulation of PRL and PDGF (PRL added 10 min prior to PDGF) is referred as P/P, while the sum of PRL and PDGF stimulation alone is referred as PRL+PDGF. For *c-fos* transactivation by P/P *versus* PRL+PDGF, $p < 0.01$. **B.** Pooled data from three such experiments, as shown in A, are plotted. The sum of fold increase caused by PRL alone and PDGF alone was set as 100%. The costimulation-induced fold increase compared with the summation was calculated. The costimulation augmented *c-fos* transactivation by $58.6 \pm 2.4\%$ vs. the summation ($p < 0.01$).