

# Up-Regulation of Urokinase-Type Plasminogen Activator by Insulin-like Growth Factor-I Depends upon Phosphatidylinositol-3 Kinase and Mitogen-activated Protein Kinase Kinase

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## ABSTRACT

Elevated levels of urokinase plasminogen activator-I (uPA) and the insulin-like growth factor-I receptor (IGF-IR) are associated with breast cancer recurrence and decreased survival. It is possible that activation of IGF-IR and elevations in uPA are mechanistically linked. Our laboratory recently showed that insulin-like growth factor-I (IGF-I) induces uPA protein and mRNA in the breast cancer cell line MDA-MB-231. We also found that IGF-IR and uPA were commonly overexpressed in primary breast cancers. In this study, we investigated the signal transduction pathway through which IGF-I regulates uPA. Phosphatidylinositol 3-kinase, mitogen-activated protein kinase kinase, and p70 kinase were inhibited with LY294002, PD98059, and rapamycin, respectively. Induction of uPA protein by IGF-I was partially inhibited by LY294002 (60% inhibition) or PD98059 (30% inhibition) but not by rapamycin. The production of uPA protein induced by IGF-I was blocked up to 90% by the tyrosine kinase inhibitor herbimycin A. Furthermore, herbimycin A suppressed the phosphorylation of AKT and Erk1/2. Next, we tested the impact of the signal transduction inhibitors on uPA gene expression. Both LY294002 and PD98059 were required to completely inhibit uPA mRNA expression, whereas each drug alone resulted in approximately 50% reduction in uPA expression. Next, using a minimal uPA-luciferase promoter construct containing the binding sites for the AP-1 and Ets transcription factors, we observed that IGF-I stimulated the uPA promoter via these sites. Furthermore, both LY294002 and PD98059 were necessary to block IGF-I-stimulated uPA-Luc activity. In summary, we conclude that IGF-I requires both phosphatidylinositol 3-kinase and mitogen-activated protein kinase kinase-dependent pathways to optimally induce uPA expression. These findings suggest that the development of drugs targeting these pathways may benefit breast cancer patients at a high risk of recurrence, such as those who have primary tumors overexpressing IGF-IR and uPA.

## INTRODUCTION

Currently, 2.6 million women in the United States are breast cancer survivors. Fortunately, early detection and better treatment options have improved the chances of surviving this disease, but recurrence of breast cancer is an unfortunate reality for some of these women. Therefore, it is imperative to find reliable prognostic indicators for recurrence and/or long-term survival. uPA<sup>2</sup> is commonly overexpressed in breast cancers and is a strong prognostic indicator for decreased patient survival rates. The reported relative risk of relapse is 21.1-fold when uPA levels are elevated in primary breast cancers (1). This is a striking elevation in risk for relapse, particularly com-

pared with other prognostic indicators such as the status of the hormone receptor and lymph node involvement, which have a relative risk of 5.8 and 3.0, respectively. Other studies (2) demonstrate that uPA levels are useful in predicting poor survival rates for node-negative breast cancer patients. This is notable because the current trend is to use axillary lymph node status to discriminate between a good and poor prognosis, despite the fact that it is not always a reliable indicator. Studies show that 30% of node-negative patients and 65% of node-positive patients will experience a relapse (3). Elevations in uPA protein do not simply characterize breast cancers but are also relevant to malignancies of the bladder, colon, lung, ovary, (4) and prostate (5). Therefore, it is critical to elucidate how uPA is regulated as a movement toward preventing cancer recurrence and improving overall survival rates for a wide range of cancer patients.

The mechanism whereby uPA influences cancer progression hinges upon its ability to regulate tumor invasion and angiogenesis. Cellular invasion is mediated by uPA through the conversion of plasminogen to plasmin, which degrades basement membranes (6). In a plasmin generation assay, the human breast cancer cells MDA-MB-231 are very efficient in converting plasminogen to plasmin (7). Plasmin activation is abolished by adding a neutralizing antibody to uPA or uPA receptor, which results in a subsequent suppression of cellular invasion (7). Furthermore, known inhibitors of plasmin suppress the degradation of basement membrane proteins (6). Plasmin production can influence invasion both directly and indirectly. Plasmin directly mediates invasion by degrading ECM proteins such as laminin, collagen IV, and fibronectin. Furthermore, plasmin stimulates invasion indirectly by activating proteases such as the MMP-2 and -9 (8), stromelysin (9), or uPA (10). Animal models further support the role of uPA in regulating invasion and metastases, *e.g.*, plasminogen-deficient mice have a significant reduction in mammary tumor metastases to the lungs compared with mice that express normal levels of plasminogen in the mammary tissue (11). Moreover, uPA overexpression results in an increase in skeletal metastasis in a prostate model (12). These studies collectively suggest that disrupting uPA could have a therapeutic benefit. In preclinical studies, the administration of synthetic uPA inhibitors such as amelioride (13) or B428 (14) reduced tumor metastases in a rat mammary model. Although provocative, these inhibitors have not gained widespread acceptance because of drug stability and cytotoxicity problems. More recently, a small molecule inhibitor of uPA was efficacious in blocking the uPA/uPA receptor system in rat and human models of breast cancer (15). The evolution of new uPA inhibitors is promising, but further work is still needed to understand how uPA is regulated in human breast cancers.

The tyrosine kinase growth factors Erb-B2 and IGF-IR are overexpressed in human breast cancers and could conceivably regulate invasion and metastasis via uPA induction. A decrease in survival and an increase in the probability of relapse are predicted by elevated levels of Erb-B2 (16) and IGF-IR (17). Overexpression of uPA in breast cancer has an analogous association; therefore, these prognostic

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<sup>2</sup> The abbreviations used are: uPA, urokinase-type plasminogen activator; Luc, luciferase; ECM, extracellular matrix; MMP, matrix metalloproteinases; IGF-I, insulin like growth factor-I; IGF-IR, IGF-I receptor; EGF, epidermal growth factor; MEK, mitogen-activated protein kinase kinase; PI3K, phosphatidylinositol 3kinase; ER, estrogen receptor; TKR, tyrosine kinase receptor; 17-AG, 17-allylamino-17-demethoxygeldanamycin.

indicators may be mechanistically linked. Studies supporting this possibility show that activation of Erb-B2 with EGF stimulates uPA mRNA in the human breast cancer cell line SkBr-3 (18). Activation of Erb-B2 also results in a significant increase in cellular invasion (18). Similarly, the stable overexpression of Erb-B2 activates uPA in human lung cancer cells (19). The possible link between IGF-IR and/or Erb-B2 with uPA was pursued in our laboratory. We found a positive association between IGF-IR and uPA in women with Stage I and II breast cancer.<sup>3</sup> However, Erb-B2 was not correlated with high uPA expression in that study. Our laboratory also demonstrated that activation of IGF-IR stimulated invasion of the breast cancer cell line, MDA-MB-231 *in vitro* (20). Coordinated with a stimulation of invasion, we observed an increase in uPA protein and mRNA. These intriguing results led us to decipher the signal transduction pathways from IGF-IR leading to uPA regulation. Very little is known about how uPA is regulated by cytokines in general. It appears that colony stimulating factor-1 activates uPA in a MEK-dependent manner in macrophages (21). However, no studies have been conducted to address how tyrosine kinase-signaling pathways regulate uPA in cancer cells.

In breast cancer cells, IGF-IR signaling promotes growth (22), inhibits cell death (23), and stimulates cellular invasion (24, 25). This combination of seemingly disparate cellular functions results from the activation of discrete second messengers. Activated IGF-IR transmits cellular signals via second messengers such as GRB10, SHC, insulin response substrate-1, and insulin response substrate-2. IGF-IR phosphorylates SHC and leads to the activation of the mitogen-activated protein kinase pathway, whereas phosphorylation of IRS-1 results in the recruitment of PI3K and the concomitant phosphorylation of AKT or activation of p70 kinase. Neutralizing antibodies, dominant negative inhibitors, or chemical-blocking agents are useful for disrupting IGF-IR. The growth of tumors derived from MDA-MB-231 cells is suppressed by neutralizing antibodies to IGF-IR (26), and we demonstrated that invasion is suppressed in the same cell line using a dominant negative inhibitor (20). We also demonstrated that inhibition of IGF-IR with a dominant negative inhibitor blocks breast cancer invasion and metastases of MDA-MB-435 cells (24). The tyrosine kinase inhibitor, herbimycin A, inhibits IGF-IR signaling by stimulating receptor degradation in the MCF-7 human breast cells (27). Each of these tools has the potential to disrupt IGF-IR and, therefore, to block downstream signaling that eventually leads to the uPA.

Both AKT and MEK are reportedly elevated in primary breast cancers, although how these second messengers are activated and the consequence of signal amplification remains unknown. MEK is overexpressed and hyperphosphorylated in primary breast tumors, as well as in metastatic cancers (28). In addition, activated MEK is higher in MDA-MB-231 cells than in the less invasive MCF-7 cells (29). PI3K/AKT is also reportedly activated in breast cancers. Some human breast cell lines have higher AKT kinase activity compared with the nonneoplastic breast epithelial MCF-10A (30). Downstream from PI3K and MEK lies the AP-1 transcription complex. Interestingly, the DNA-binding activity of AP-1 is greater in MDA-MB-231 compared with MCF-7 cells growing in logarithmic phase (31). Moreover, this study showed that AP-1 is activated by select growth factors. AP-1 activity is induced by signaling through the IGF-IR or EGF receptor but not through platelet-derived growth factor receptor or ER (31). On the basis of these data, targeting tyrosine kinase receptors relevant to breast cancer could block cytoplasmic and nuclear signal transduction.

In this study, we used specific signal transduction inhibitors to determine which pathway(s) are necessary for uPA activation by IGF-I. We found that IGF-I stimulates uPA and cellular invasion in a PI3K- and MEK-dependent manner. In contrast, IGF-I does not require p70 kinase to control these events. Herbimycin A is also very effective in attenuating uPA induced by IGF-I. Therapeutic agents that converge on these pathways may be beneficial to breast cancer patients that are at a high risk of recurrence, such as those who have primary tumors that overexpress IGF-IR and uPA.

## MATERIALS AND METHODS

**Chemicals.** The PI3K inhibitor, LY294002, was purchased from Sigma Chemical Co. (St. Louis, MO). The MEK inhibitor, PD98059, was purchased from New England Biolabs (Beverly, MA). The p70 kinase inhibitor, rapamycin, was purchased from Calbiochem (Cambridge, MA). Herbimycin A was also purchased from Calbiochem.

**Cell Lines.** The MDA-MB-231 and MCF-7 cell lines were purchased from American Type Culture Collection (Rockville, MD) and maintained in 10% FCS DMEM F-12. Each cell line tested negative for *Mycoplasma* contamination. Cellular viability was determined using the MTS assay as described previously (23) by our laboratory using a commercially available kit (Promega, Madison, WI).

**uPA ELISA.** Cells were plated at a density of  $5 \times 10^5$  in a 12-well dish. Before exposure to IGF-I, they were rinsed with PBS and serum starved for 24 h. IGF-I (long R3; Diagnostic Systems Laboratories, Inc., Webster, TX) was added at a concentration of 100 ng/ml in serum-free DMEM F12. The conditioned media was collected 24 h after exposure to IGF-I and stored at  $-70^\circ\text{C}$ . The uPA assay was performed according to the manufacturer's instructions (Imubind uPA ELISA Kit; American Diagnostica, Inc., Greenwich, CT). In preparation for uPA analyses, the conditioned media was diluted  $10\times$  in sample buffer (provided with the kit). We modified the uPA assay for the MCF-7 cells because we were unable to detect uPA on cells growing on plastic. Alternatively, MCF-7 cells plated on fibronectin express uPA (32). We plated  $5 \times 10^3$  cells on collagen I-coated 96-well plates (Collaborative Biomedical Products, Bedford, MA), allowed the cell to attach for 24 h, and then serum starved the cells for the next 24 h. IGF-I (100 ng/ml; long R3)-supplemented media with or without signal transduction inhibitors was provided to the MCF-7 cells for 24 h. The conditioned media was not diluted before uPA analyses. The MDA-MB-231 and MCF-7 cells were pretreated with herbimycin A (0, 0.02, 0.1, 0.50, and 1.0  $\mu\text{g/ml}$ ) for 24 h before addition of IGF-I. DMSO was administered as a vehicle control.

**Northern Blot.** Cells were plated at a density of  $2 \times 10^6$  in a 150-mm dish. Total RNA was isolated using Trizol (Life Technologies, Inc.). A total of 10  $\mu\text{g}$  of RNA was separated on a 1% agarose gel. RNA was transferred using a TurboBlotter (Schleicher & Schuell, Keene, NH). The uPA probe was purchased from Research Genetics (Huntsville, AL). The probe was labeled using a RadPrime kit (Life Technologies, Inc.), and the blots were hybridized using Quik Hyb (Stratagene, La Jolla, CA). Blots were hybridized and washed under high stringency conditions.

**uPA-Luc Assay.** MDA-MB-231 cells were stably transfected with a uPA-Luc construct or the vector control PGL-2 (kindly provided by Dr. Michael Coleman, Millipore, Bedford, MA). The cells were incubated with IGF-I for 24 h, harvested with lysis buffer, and then stored at  $-70^\circ\text{C}$ . Luc activity was determined using a Dual-Luciferase Reporter Assay system (Promega). Renilla was stably cotransfected into the cells to serve as an internal control for the Dual-Luciferase reporter system.

**Western Blots.** Cells ( $5 \times 10^6$ ) were plated in T150 culture flasks, and the next day the cells were rinsed with PBS and placed in serum-free DMEM-F12 for 24 h. The next day, cells were treated without or with IGF-I (10 ng/ml). When signal transduction inhibitors were used, the cells were pretreated for 10 min before the addition of IGF-I. DMSO was administered as a vehicle control for the experiments that included the signal transduction inhibitors. The concentrations of the inhibitors were the same as described in the uPA ELISA section. In addition, cells were plated on collagen I to be consistent with the uPA analyses. Cells were harvested and pooled, and proteins were isolated by homogenization in ice-cold lysis buffer [1% Triton X-100; 2 mM EDTA; 1 mM  $\text{Na}_3\text{VO}_4$ ; 20 mM NaF; 50  $\mu\text{M}$   $\text{Na}_2\text{MoO}_4$ ; 20  $\mu\text{g/ml}$  aprotinin; 20  $\mu\text{g/ml}$

<sup>3</sup> S. E. Dunn, M. V. Iacocca, J. V. Torres, P. M. Glazer, J. C. Barrett, and B. G. Haffty. Overexpression of urokinase plasminogen activator (uPA) correlates with elevations in the insulin-like growth factor-1 receptor (IGF-IR) and the ER in primary breast cancers, submitted for publication.

leupeptin; and 15  $\mu\text{M}$  (4-aminophenyl)-methanesulfonyl fluoride in 20 mM HEPES (pH 7.4)]. Western blot analysis was performed as described previously (33) with the exception of the following modifications. AKT, phospho-AKT, and phospho-Erk1/2 were detected using polyclonal antibodies (New England Biolabs) according to the manufacturer's instructions.

**Invasion Assays.** Cellular invasion was assessed using a modified Boyden chamber (Neuro Probe Inc., Cabin John, MA). Membranes were coated with collagen IV (25  $\mu\text{g}/\text{ml}$ ) in 10 mM acetic acid for 1 h at room temperature. The lower chamber was filled with DMEM-F12 supplemented with IGF-I 10 ng/ml (Collaborative Biomedical Products, Bedford, MA). Invasion assays were performed according to Doerr *et al.* (25). MDA-MB-231 cells ( $2 \times 10^4$  cells/well) were allowed to invade for 4 h. Cells were exposed to signal transduction inhibitors (30  $\mu\text{M}$  LY294002; 20  $\mu\text{M}$  PD98059; and 0.5 nM rapamycin) for 10 min before the initiation of the study.

## RESULTS

**IGF-I Stimulates uPA Protein in ER Positive and ER Negative Breast Cancer Cells.** Breast cell lines were screened to determine whether IGF-I commonly induced uPA. IGF-I induced uPA in MDA-MB-231 > MDA-MB-436 > HS 578T > MDA-MB-157 cells (Fig. 1A). None of the cell lines that responded were ER positive. This result was unexpected because of the fact that we observed that uPA is frequently expressed in primary breast cancers that are either ER positive or ER negative.<sup>3</sup> It is conceivable that epithelial cell interactions with ECM protein may be necessary for optimal activation of uPA by IGF-I. To test this idea, we plated the ER positive breast cell line MCF-7 on plates coated with the interstitial matrix protein, collagen I. IGF-I induced uPA in the MCF-7 cells, whereas estrogen had no effect (Fig. 1B). These studies illustrate that IGF-I stimulates uPA in both ER positive and ER negative breast cell lines.

**Disruption of IGF-IR Signaling by Ly293002, PD98059, or Herbimycin A Results in a Significant Decrease in uPA Protein Production.** IGF-I binding to IGF-IR produces a cascade of downstream signaling events that regulate cellular invasion. To determine the second messengers necessary for transmitting the IGF-I signal to uPA, a series of signal transduction inhibitors were studied for their ability to inhibit the regulation of uPA by IGF-I in MDA-MB-231 cells. The PI3K inhibitor LY294002 suppressed IGF-I induction of uPA in a time-dependent manner (Fig. 2). uPA was inhibited by approximately 60% at the 24-h time point. PD98059 and rapamycin were next tested to determine their effect on the induction of uPA by IGF-I. Treatment with PD98059 resulted in a 30% suppression of uPA after 24 h (Fig. 3A). In contrast, rapamycin had no significant effect. uPA was also inhibited in the MCF-7 cells by 70% and 56% with LY294002 and PD98059, respectively (Fig. 3B). The suppression of uPA was not attributable to cell death because the number of viable cells remained the same between treatment groups after 24 h (data not shown).

To confirm that IGF-I was activating the PI3K pathway, we stimulated MDA-MB-231 cells with IGF-I (10 ng/ml) for 0, 2, 5, and 10 min and observed an increase in phospho-AKT (Fig. 4A). Furthermore, we demonstrated that LY294002 inhibited the phosphorylation of AKT to confirm PI3K inactivation (Fig. 4B). In contrast, rapamycin and PD98059 did not block AKT phosphorylation (Fig. 4, C and D, respectively). We also examined the regulation of MEK by evaluating nuclear phospho-Erk1/2. IGF-I stimulated the phosphorylation of Erk1/2 compared with untreated control cells (Fig. 5, Lane 2 versus Lane 1). PD98059 reversed the phosphorylation of Erk1/2 by IGF-I (Lane 4 versus Lane 2), whereas LY294002 and rapamycin had no effect (Fig. 5, Lanes 3 and 5, respectively). Next, we turned to the tyrosine kinase inhibitor, herbimycin A, to assess whether this compound had inhibitory effects on uPA. Herbimycin A also inhibited IGF-I-stimulated uPA production by 50–90% without adversely af-

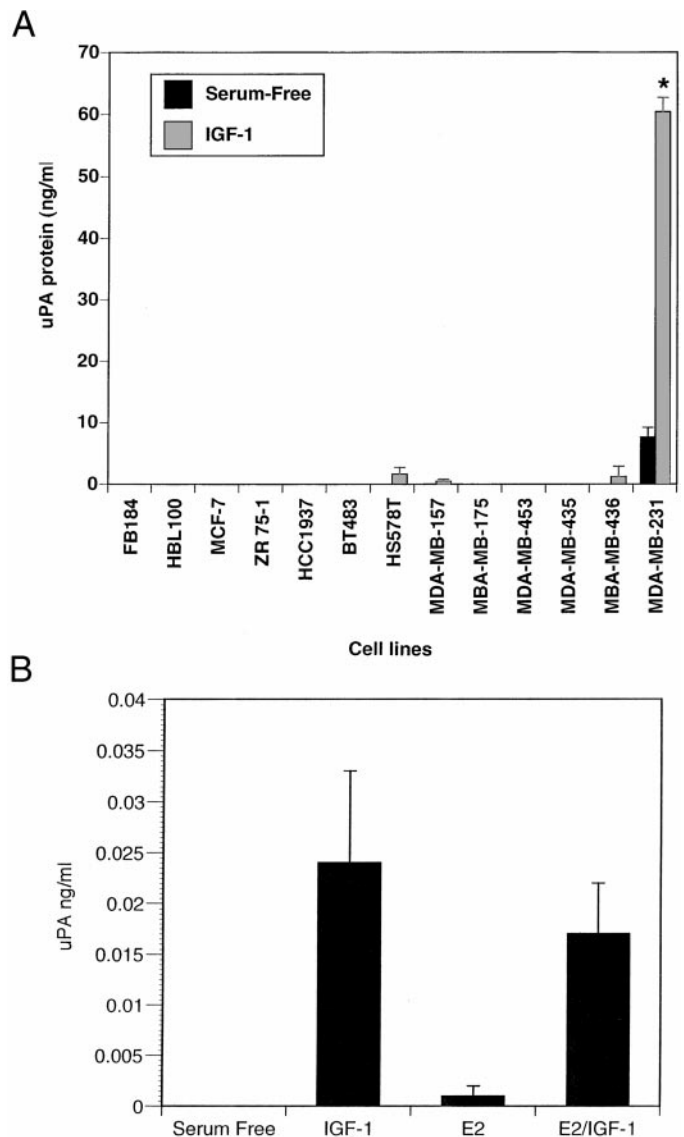


Fig. 1. Production of uPA protein in breast cancer cell lines. A, induction of uPA protein in breast cells treated with IGF-I (long R3; 10 ng/ml) for 24 h. IGF-I significantly induced uPA protein in the MDA-MB-231 cells compared with the serum-free-treated cells. There was an induction in the HS578T, MDA-MB-157, and MDA-MB-436 cell lines. Statistical significance was determined by the Student *t* test; \*,  $P < 0.05$ . B, uPA was induced in MCF-7 cells when plated on a collagen I-coated plate. Estrogen alone had a minimal effect on uPA production. In addition, estrogen plus IGF-I did not increase uPA production compared with IGF-I alone.

fecting cell survival (Fig. 6A). We also found that pretreatment with herbimycin A for 24 h blocked phosphorylation of AKT (Fig. 6B) and Erk1/2 (Fig. 6C) in the presence of IGF-I.

**Regulation uPA mRNA by IGF-I Depends on the PI3K and MEK Pathways through the AP-1 and Ets Sites on the uPA Promoter.** On the basis of our previous demonstration that IGF-I induced uPA mRNA (20), we tested whether the signal transduction inhibitors regulated uPA gene expression. We found that blocking IGF-I signaling with LY294002 or PD98059 suppressed uPA mRNA by 50% based upon densitometric quantification (Fig. 7). This approximates the degree of inhibition observed at the protein level. Interestingly, when LY294002 and PD98059 were added together, uPA mRNA was suppressed completely. We extended this observation by testing the signal transduction inhibitors in a uPA-Luc assay. A minimal uPA promoter containing the Ets and AP-1 sites was linked to a Luc reporter (Fig. 8A). IGF-I stimulated uPA-Luc activity



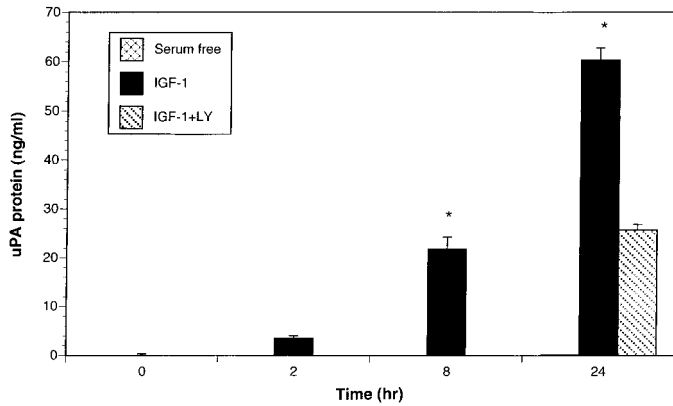


Fig. 2. Regulation of uPA protein by IGF-I in the absence or presence of the PI3K inhibitor, Ly294002. MDA-MB-231 cells were treated without (□) or with (■) IGF-I. IGF-I increased uPA protein production over a 24-h period. The addition of Ly294002 (30  $\mu$ M) to IGF-I-treated cells inhibited uPA induction. The amount of uPA inhibition by Ly294002 was statistically significant at the 8- and 24-h time points. In the presence of Ly294002, uPA protein was below the level of detection at the 2- and 8-h time points. Statistical significance was determined by the Student *t* test; \*,  $P < 0.05$ ;  $n = 4$  replicates.

6-fold (Fig. 8B) compared with the PGL-2 negative control (Fig. 8C). The reporter activity of uPA-Luc was inhibited with Ly294003 (39%) or PD98059 (55%). Both inhibitors resulted in a 97% inhibition (Fig. 8B).

**Cellular Migration Is Suppressed by Inhibiting PI3K and MEK.** The functional consequence of blocking IGF-I signaling in the MDA-MB-231 cells was addressed using the Boyden Chamber migration assay. IGF-I stimulated the cells to migrate across a collagen IV-coated membrane (Fig. 9). Cellular migrate was inhibited by >90% with LY294002 or PD98059 but not by rapamycin. These data demonstrate that suppressing IGF-I signaling via the PI3K or MEK pathways dramatically alters the ability of breast cancer cells to migrate.

## DISCUSSION

In this study, we found that IGF-I stimulates uPA protein and mRNA in a PI3K- and MEK-dependent manner. IGF-I increased the protein production of uPA in both ER positive and negative breast cancer cells. We also demonstrated that the ansamycin antibiotic, herbimycin A, inhibited IGF-I signal transduction via PI3K and MEK. Moreover, herbimycin A blocked the production of uPA stimulated by IGF-I. These data suggest that derivatives of herbimycin A may become valuable chemopreventive agents, particularly for women considered to be at high risk, *e.g.*, there is an association between elevated serum IGF-I and the development of breast cancer in premenopausal women (34). Our data also provide support for inhibiting uPA by disrupting tyrosine kinase signaling that could be used to prevent the recurrence of breast cancer.

IGF-I induces not only uPA but also other ECM proteases, *e.g.*, IGF-I also induces MMP-9 mRNA and protein (35). We showed previously (24) that disruption of IGF-IR suppressed invasion and metastases of the human breast cancer cell line MDA-MB-435, although we demonstrated in this study that IGF-I does not induce uPA in these cells. We suspect that IGF-I/IGF-IR signaling regulates other ECM proteases such as the MMPs. Induction of uPA and MMP-9 by IGF-I could influence tumor progression by stimulating invasion. Beyond their previously described functions as ECM-degrading proteins, MMP-9 and uPA also exert their effects on cellular growth via degradation of IGFBP-1 (35) and IGFBP-3 (36), respectively. IGFBP-1 is a potential physiological substrate for human stromelysin-3, which is a member of the MMP family of proteases (37).

Cleavage of IGFBP-1 and IGFBP-3 releases IGF-I making it available to bind IGF-IR. Furthermore, an autocrine loop for IGF-I could increase the malignant potential of cancer cells by stimulating proliferation and invasion. The human prostate cancer cells PC-3 are highly invasive and have an autocrine loop for IGF-I, whereas the modestly invasive Du145 cells lack this characteristic (38). The PC-3 cells also produce copious amounts of uPA. IGF-IR-neutralizing antibodies decrease uPA production in these cells (38). Furthermore, the production of uPA results in cleavage of IGFBP-3, release of IGF-I, and cellular invasion (38). Hence, there is potential cross-talk between the activation of proteases by IGF-I and the concomitant cleavage of IGF-I/IGFBP complexes that may facilitate the progression of cancer. IGF-I signaling has the potential to influence local cancer invasion by up-regulating ECM proteases such as uPA, MMP, and stromelysin. The cellular response to an increase in ECM proteases is 2-fold. First, the cells invade into the stroma by secreting ECM-degrading proteases. Secondly, the release of ECM proteases such as uPA would

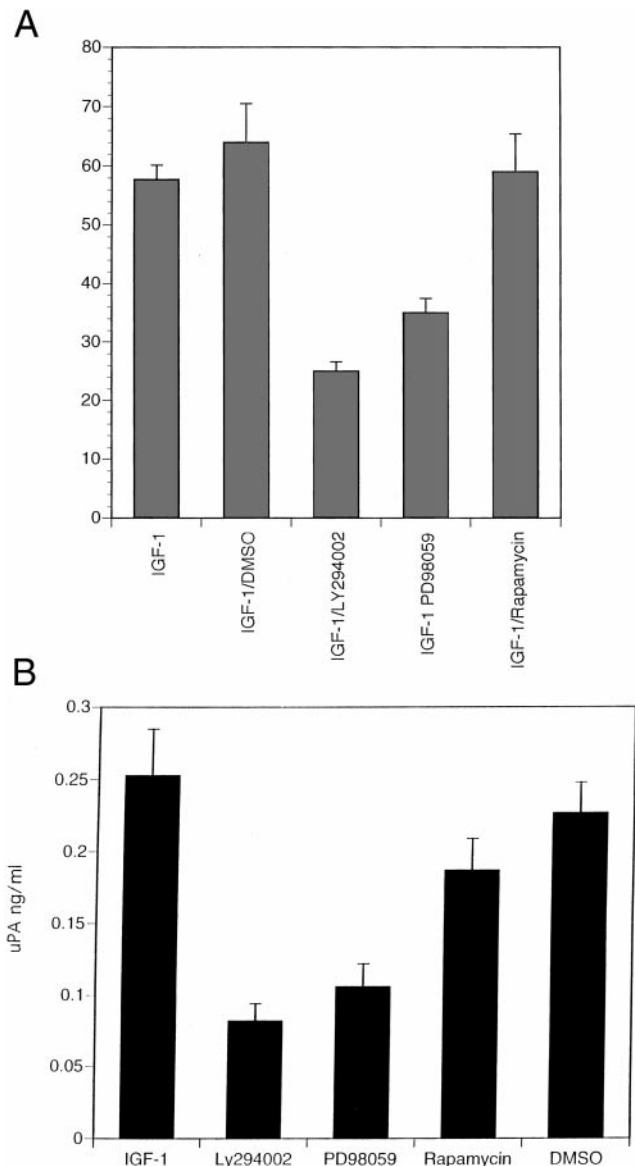


Fig. 3. Regulation of uPA protein by inhibition of PI3K, MEK, or p70 kinase by Ly294002, PD98059, or rapamycin, respectively. MDA-MB-231 (A) or MCF-7 (B) cells were pretreated with LY294002 (30  $\mu$ M), PD98059 (20  $\mu$ M), rapamycin (0.5 nM), or the DMSO (vehicle) for 30 min before the addition of IGF-I (10 ng/ml). uPA protein was suppressed by Ly294002 and PD98059 but not by rapamycin or DMSO. Statistical significance was determined by the Student *t* test; \*,  $P < 0.05$ ;  $n = 4$  replicates.

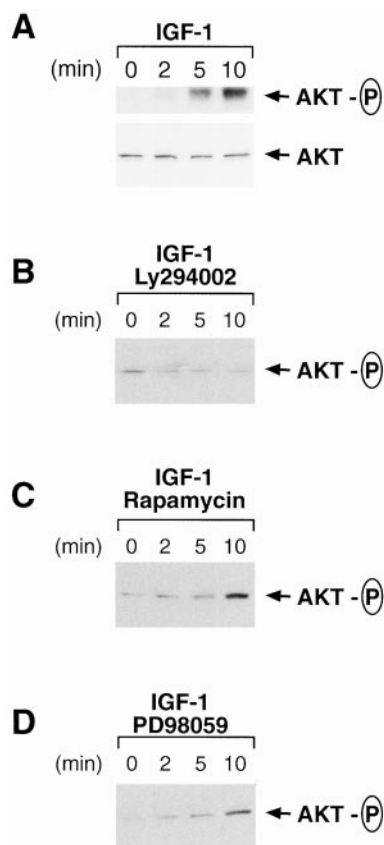


Fig. 4. A, induction of phospho-AKT by IGF-I. MDA-MB-231 cells were treated with IGF-I for 0, 2, 5, and 10 min. AKT was phosphorylated in a time-dependent manner when the cells were stimulated with IGF-I. To ensure that equal amounts of protein (10  $\mu$ g) were loaded into each lane, the blot was reprobed for AKT protein expression (bottom panel). B, Phospho-AKT (AKT-P) was inhibited by LY294002. IGF-I (10 ng/ml) was added to MDA-MB-231 cells for 0, 2, 5, and 10 min in the presence of 30  $\mu$ M LY294002, (C) rapamycin (0.5 nM), or (D) PD98059 (20  $\mu$ M), and then the cell lysates were evaluated for an inhibition of phospho-AKT. LY294002-inhibited IGF-I stimulated phosphorylation of AKT. In contrast, IGF-I stimulated the phosphorylation of AKT alone or in the presence of rapamycin or PD98059.

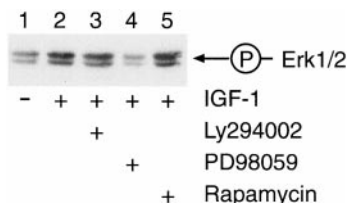


Fig. 5. Induction of phospho-Erk1/2 by IGF-I. MCF-7 cells were treated without or with LY294002, PD98059, or rapamycin for 60 min. Less phospho-Erk1/2 was found in untreated cells compared with those supplemented with IGF-I (Lane 1 versus Lane 2, respectively). PD98059 significantly suppressed phospho-Erk1/2 in the presence of IGF-I (Lane 4), whereas LY294002 and rapamycin had no effect (Lanes 3 and 5, respectively). A total of 50  $\mu$ g of protein was loaded into each lane.

increase the bioavailability of IGF-I by cleaving the IGF/IGFBP complex and initiate the production of ECM proteases again.

IGF-I regulates many cellular functions that are important to the development of breast cancer such as mitogenesis, differentiation, apoptosis, and invasion. We are just beginning to appreciate that each cellular process has a unique second messenger cascade for transmitting IGF-I-induced signaling. Our study showed that IGF-I mediates invasion through PI3K and MEK. In contrast, mitogenesis induced by IGF-I requires PI3K but is independent of the MEK pathway (39). The PI3K pathway also negatively controls apoptosis MCF-7 cells (30). Conversely, inhibition of AKT allows apoptosis to ensue even in the

presence of the antiapoptotic hormones, estradiol or IGF-I (30). PI3K/AKT inhibitors may become important for cancer chemotherapy by blocking proliferation, inducing apoptosis, and inhibiting invasion. Two novel PI3K/AKT inhibitors were recently discovered and hold promise for such applications. Phospholipid inositol 1,3,4,5,6-pentakisphosphate and inositol 1,4,5,6-tetrakisphosphate are PI3K inhibitors that block proliferation of MCF-7 cells in the presence of IGF-I (40). Furthermore, the inhibitors are comparable with LY294002 in attenuating constitutively active PI3K in the ovarian cancer cells SKOV-3 (40). These studies collectively suggest that the PI3K pathway is emerging as a conduit for the stimulation of cell growth and invasion along with a simultaneous suppression of apoptosis.

One candidate approach to developing anticancer drugs is to inhibit TKRs because oncogenes, such as activated *IGF-IR*, *Erb-B2*, and *c-met*, are commonly overexpressed in human cancers. Ansamycin antibiotics inhibit several oncogenic proteins; hence, they are attractive anticancer drugs, particularly for breast cancers with IGF-IR and/or Erb-B2 overexpression. These compounds not only decrease IGF-IR but also inhibit HSP90 (41), Raf-1, Erb-B2 (42), and mutant p53 (43). We demonstrated that the tyrosine kinase inhibitor herbimycin A significantly blocks induction of uPA protein by IGF-I. Our results are consistent with reports demonstrating that PI3K/AKT (44) and MEK (45) pathways are inhibited with these compounds along a subsequent suppression of AP-1 activity (46). Our research also complements a recent report (47) demonstrating that the geldanamycin family of ansamycin antibiotics inhibit uPA stimulated by c-Met,

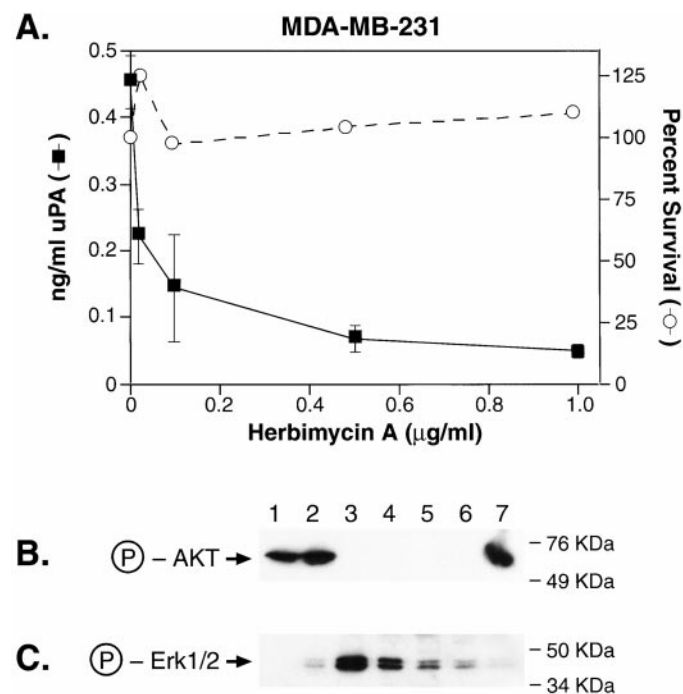


Fig. 6. Stimulation of uPA by IGF-I was inhibited with herbimycin A in a dose-dependent manner. A, MDA-MB-231 cells were pretreated with herbimycin A (0, 0.02, 0.1, 0.5, and 1.0  $\mu$ g/ml) for 24 h before the addition of IGF-I. Both cell lines responded to herbimycin A by a decrease in uPA production when IGF-I was added. There was no adverse effect on cell viability. Each experiment was performed in replicates of four to six. B, herbimycin A inhibited phosphorylation of AKT (Lanes 1–5 contained samples treated with herbimycin A at concentrations of 0, 0.02, 0.1, 0.5, and 1.0  $\mu$ g/ml, respectively). Cell lysates from NIH3T3 cells treated without (Lane 6, negative control) or with platelet-derived growth factor (Lane 7, positive control). Right, a broad range molecular weight standard. A total of 50  $\mu$ g of protein was loaded into each lane. C, Erk1/2 phosphorylation was blocked in the presence of herbimycin A (0, 0.02, 0.1, 0.5, and 1.0  $\mu$ g/ml in Lanes 3–7). MCF-7 nuclear extracts were isolated from cells treated without (Lane 1, negative control) or with IGF-I (Lane 2, positive control). Right, a broad range molecular weight standard. A total of 50  $\mu$ g of protein was loaded into each lane.

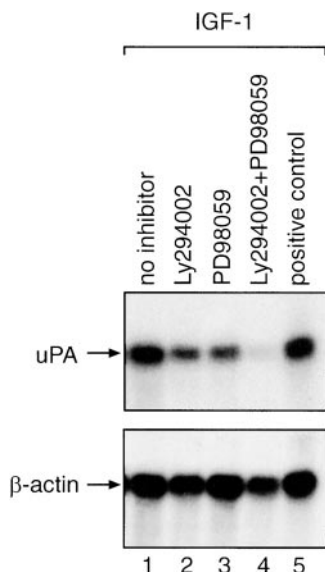


Fig. 7. Inhibition of uPA mRNA by LY294002 and PD98059. MDA-MB-231 cells were treated with IGF-I (10 ng/ml) for 24 h in the absence (Lane 1) or presence (Lane 2) of LY294002 (30  $\mu$ M), PD98059 (20  $\mu$ M; Lane 3), or LY294002 plus PD98059 (Lane 4). LY294002 and PD98059 inhibited uPA mRNA individually, although when added together, uPA mRNA was almost undetectable. IGF-I was shown previously to induce uPA as early as 90 min, thus this RNA was run as a positive control for the detection of uPA (Lane 5). To ensure that the RNA was loaded equally, the samples were probed for  $\beta$ -actin.

which is a TKR but not commonly altered in breast cancer. The ansamycin antibiotics were identified by the National Cancer Institute under a program that screened over 1000 compounds for the ability to inhibit uPA proteolysis by c-Met. One of the key observations made by this effort was that the dose of geldanamycin inhibitor needed to block the conversion of plasmin to plasminogen was nine orders of magnitude lower than the concentration that inhibited cell growth (47). Likewise, we did not observe an inhibition of cell growth at the concentrations that inhibited IGF-I stimulation of uPA. Geldanamycin and herbimycin A are not suitable compounds for clinical use because of the overt side effects observed in preclinical studies. The second-generation ansamycin antibiotic 17-AG does not have the associated side effects and is under investigation as an anticancer drug. The antiproliferative activity of 17-AG against MCF-7 and SKBR-3 cells (41) suggest one mechanism whereby this chemical could serve as an anticancer drug. Our data suggest that the ansamycin antibiotics potentially have efficacy against cancer invasion. In addition, our study suggests that inhibition of invasion may be different from inhibition of cell growth; hence, combination therapies may prove to be most effective in treating cancer. The efficacy of 17-AG in treating human cancers is presently in Phase I clinical trials (48).

Unique signal transduction pathways may lead to the activation of ECM proteases such as uPA and MMP. Several reports (review Ref. 49) indicate that the MMPs are positively regulated by the AP-1 transcription complex. Unlike the uPA promoter, the *MMP-9* gene lacks the PEA3/ets site but has binding sites for Sp1,  $\text{Nf-}\kappa\text{B}$ , and Rb (50). The *uPA* and *MMP-1* genes are similar because they both have an AP-1 and PEA3/Ets site. Furthermore, PEA3 and AP-1 synergistically activate the transcription of MMP-1 (51). By understanding the signal transduction pathways used by TKRs, we will begin to elucidate how growth factors transmit intracellular signaling to ultimately influence cancer invasion. A recent study (52) showed that overexpression of Erb-B2 conferred PI3K-dependent invasion. Similarly, EGF stimulates SKBr-3 cells to be invasive in a Matrigel assay with a correlative increase in uPA, MMP-1, and MMP-9 (18). EGF con-

trols uPA mRNA expression by activating both the AP-1 and Ets transcription factors. Mutation deletions of the uPA promoter at the AP-1 and Ets transcription factor-binding sites inhibited EGF-induced uPA (53). We showed that inhibition of the PI3K and MEK pathways

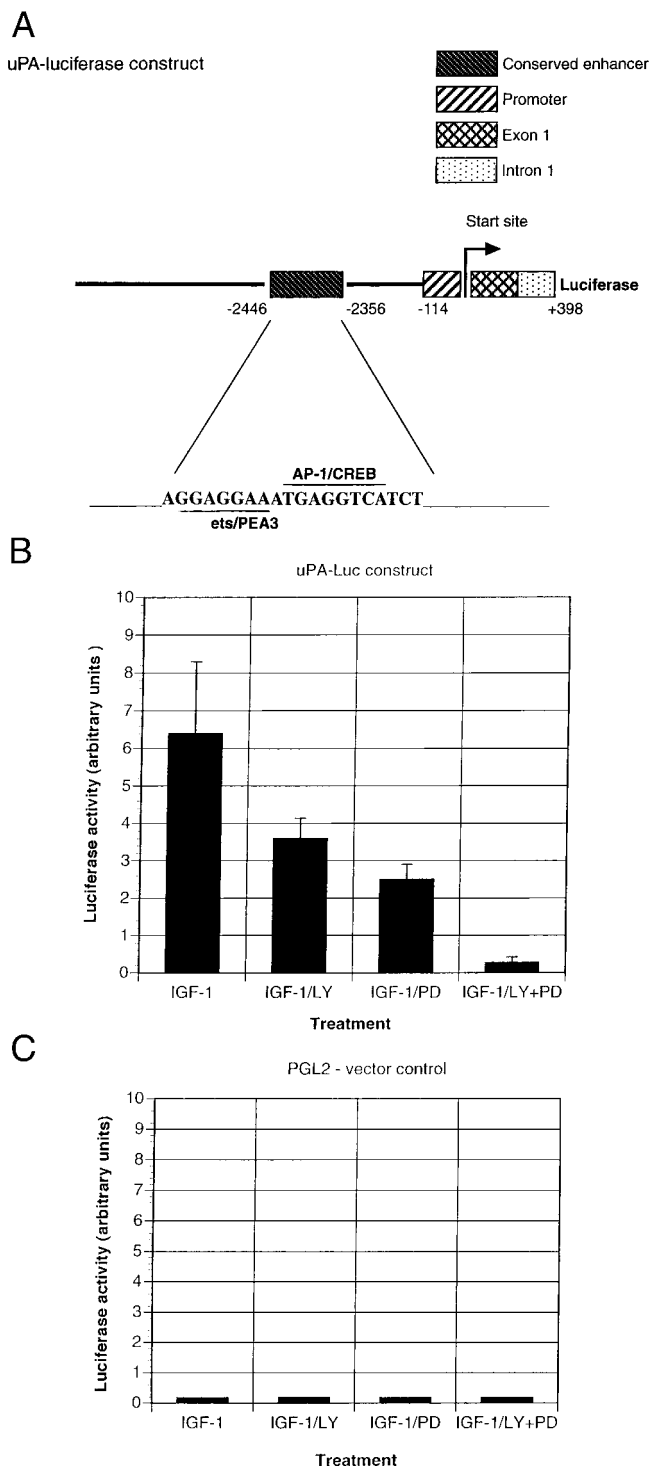


Fig. 8. LY294002 and PD98059 synergistically inhibit IGF-I-stimulated uPA-chloramphenicol acetyltransferase activity. A, the uPA-Luc construct was stably transfected into MDA-MB-231 cells. Comparisons made with the PGL-2 vector served as a negative control. Signal transduction inhibitors were introduced 30 min before the addition of IGF-I (10 ng/ml). B, IGF-I stimulation of uPA-Luc activity was partially inhibited with LY294002 or PD98059, whereas there was no effect on the PGL-2 control reporter. C, the addition of both LY294002 and PD98059 suppressed uPA-chloramphenicol acetyltransferase activity to levels comparable with the negative control. Experiments were performed in replicates of six. Statistical significance was achieved if the  $P < 0.05$ , based upon a Student *t* test.



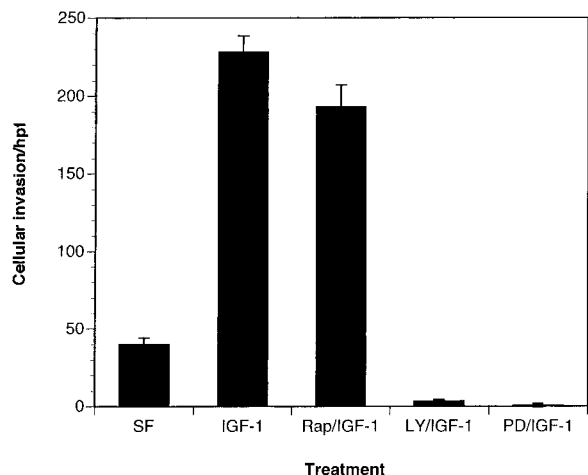


Fig. 9. Effect of signal transduction inhibitors on IGF-I stimulated migration of MDA-MB-231 cells. IGF-I stimulated the migration of MDA-MB-231 cells through a collagen IV-coated membrane compared with the serum-free control (\*). LY294002- or PD98059-blocked IGF-I stimulated migration by <90% (\*\*), compared with IGF-I alone. In contrast, rapamycin had no effect on cellular migration. Cellular migration was measured by taking the average number of cells that invaded within a high powered field ( $\times 200$ ). Each sample was analyzed in replicates of six.

resulted in an additive suppression of uPA mRNA in the presence of IGF-I. Hence, a composite Ets/AP-1 motif is also required for optimal uPA promoter activity in the presence of IGF-I. In summary, our study demonstrates that IGF-I regulates uPA protein and mRNA. These data suggest a unifying concept that TKRs regulate uPA by the convergence of the PI3K and MEK pathways.

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