Tamoxifen Interferes with the Insulin-like Growth Factor I Receptor (IGF-IR) Signaling Pathway in Breast Cancer Cells

Marina A. Guvakova and Ewa Surmacz

Kimmel Cancer Institute, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

Abstract

The insulin-like growth factor I receptor (IGF-IR) is involved in the control of breast cancer cell growth. The cytosolic activity of tamoxifen (Tam), a nonsteroidal antiestrogen, is partially mediated through interference with IGF-IR-dependent proliferation, yet the effects of Tam on IGF-IR intracellular signaling have never been elucidated. Consequently, we investigated how Tam modifies the IGF-IR signaling pathway in estrogen receptor-positive MCF-7 breast cancer cells and in MCF-7-derived clones overexpressing either the IGF-IR (MCF-7/IGF-IR cells) or its major substrate, IRS-1 (MCF-7/IRS-1 cells). MCF-7/IGF-IR and MCF-7/IRS-1 cells exhibit greatly reduced estrogen growth requirements but retain estrogen receptors and express sensitivity to antiestrogens comparable to that in the parental cells. In all tested cell lines, regardless of the amplification of IGF signaling, a 4-day treatment with 10 nm Tam produced a similar cytosolic effect. In MCF-7 and MCF-7/IGF-IR cells, growth inhibition by Tam was associated with the reduced tyrosine phosphorylation of the IGF-IR in the presence of IGF-I; however, the basal level of the IGF-IR remained unaffected. Moreover, Tam inhibited both basal and IGF-I-induced tyrosine phosphorylation of IRS-1, which was accompanied by down-regulation of IRS-1-associated phosphatidylinositol 3'-kinase activity and reduced IRS-1/growth factor receptor-bound protein 2 (GRB2) binding. In contrast, under the same treatment, tyrosine phosphorylation of Src-homology/collagen proteins (SHC); another substrate of the IGF-IR and SHC/GRB2 binding were elevated. The protein levels of the IGF-IR and IRS-1 were not modified by Tam, whereas SHC protein expression was either not affected or moderately decreased by the treatment.

In summary, this work provides the first evidence that in MCF-7 cells, cytosolic effects of Tam are associated with the modulation of IGF-IR signaling, specifically with: (a) down-regulation of IGF-RI-induced tyrosine phosphorylation of the IGF-IR; (b) inhibition of IRS-1/phosphatidylinositol 3'-kinase signaling; and (c) up-regulation of SHC tyrosine phosphorylation and increased SHC/GRB2 binding. It is hypothesized that dephosphorylation of IRS-1 could be a major contributing factor in Tam cytosolic activity.

Introduction

The activation of the IGF-IR, through a paracrine, autocrine, or endocrine mechanism, appears to play a critical role in the regulation of breast cancer cell growth (1). The IGF-IR levels are significantly higher in breast cancer than in normal breast tissue or benign tumors (1–3). The IGFs are potent mitogens for cultured breast cancer cells, and their expression has been documented in the epithelial and/or stromal component of breast tumors (1). In primary breast cancer, a correlation has been found between tumor size, the levels of IRS-1 (a cellular substrate of the IGF-IR), and recurrence of the disease (4). In MCF-7 breast cancer cells, the overexpression of either IRS-1 (5), the IGF-IR (6), or IGF-II (7) have been shown to reduce estrogen growth dependence. On the other hand, it has been demonstrated that blockade of IGF-IR signaling with, for instance, anti-IGF-IR antibodies (1), antisense RNA to the IGF-IR (8), and antisense oligodeoxynucleotides to IRS-1 (5) restricts breast cancer cell growth in vitro or in vivo.

The activation of the IGF-IR tyrosine kinase results in the stimulation of diverse intracellular pathways involving different signaling substrates (9). The best characterized substrates of the IGF-IR are IRS-1 and SHC. IRS-1 is a docking protein that, upon tyrosine phosphorylation by the IGF-IR, recruits several effector proteins through SH2-type interactions. For instance, IRS-1 binds and activates PI-3 kinase and SYP phosphatase as well as stimulates Ras/MAP pathway through the binding of GRB-2/SOS complexes (9). Moreover, IRS-1 has been found to interconnect with JAK-STAT (10) and integrin signaling pathways (11). SHC proteins are substrates of most tyrosine kinase receptors, many nonreceptor kinases and certain phosphatases (12, 13). Tyrosine phosphorylated SHC, similar to IRS-1, may activate Ras/MAP signaling cascade through the GRB2/SOS complex (12).

Tam, a nonsteroidal antiestrogen with partial agonist activity, is commonly used in adjuvant therapy in breast cancer management (14). Tam inhibits ER-dependent growth but also interferes with polypeptide growth factor signaling (14). The known effects of Tam or its derivative 4-OH-Tam on the IGF system in breast cancer cells include: inhibition of IGF-I stimulated growth (14, 15), modulation of IGFBP expression (1), reduced secretion of autocrine IGF (16), down-regulation of plasma levels of IGF-I in breast cancer patients (17), and decreased levels of IGF-I binding sites (18, 19). The interaction of Tam with the IGF-IR signaling pathway has not been characterized, partly because of the lack of adequate cellular models. Here, we investigated this aspect of Tam action using ER-positive MCF-7 cells as well as different MCF-7-derived cell lines overexpressing the elements of IGF-IR signaling.

Materials and Methods

Cell Lines and Cell Culture Conditions. MCF-7 cells were routinely grown in DMEM:F12 (1:1) containing 5% calf serum (6). In the experiments requiring estrogen-free conditions, the cells were cultured in phenol red-free DMEM containing 0.5 mg/ml BSA, 1 mM FeSO4, and 2 mM L-glutamine (PRF-SFM; Ref. 6).

MCF-7/IGF-IR, clones 12 and 15, and MCF-7/IRS-1, clone 3 were developed by stable transfection with the expression vectors pCDNA3/IGF-IR and CMV-IRS-1, respectively, and were characterized in detail previously (5, 6). The clones were maintained in culture for a maximum of 3 months in growth medium supplemented with 200 μg/ml G418.
Cell Growth Assay. Cells (1 × 10^5) were plated in 24-well plates in DMEM:F12 (1:1) containing 5% calf serum. The next day, designed as day 0 of the experiment, the cells were shifted to either PRF-SFM or PRF-SFM supplemented with 0.1–100 nM Tam. For each cell line, the number of cells at day 0 was taken as 100% (control). The relative increase (percentage over control) in cell number was determined after 4 days of Tam treatment.

Western Blotting and Immunoprecipitation. The levels of the IGF-IR, IRS-1, SHC, as well as tyrosine phosphorylation of these proteins, were measured by Western blotting. The protein lysates (250–500 µg) were obtained as previously described (6) and immunoprecipitated with the following antibodies: for IGF-IR, anti-IGF-IR monoclonal antibody alpha-IR3 (Oncogene Science); for IRS-1, anti-IRS-1 polyclonal antibody (UBI); and for SHC, anti-SHC polyclonal antibody (Transduction Laboratories). The immunoprecipitates were resolved by PAGE, and the IGF-IR, IRS-1, or SHC proteins were immunodetected with the following antibodies: for IRS-1, anti-IRS-1 polyclonal antibody (UBI); for IGF-IR and its Mr 200,000 precursor (20), anti-IGF-IR polyclonal antibody (Santa Cruz); and for SHC, anti-SHC monoclonal antibody (Transduction Laboratories). Tyrosine phosphorylation of the above proteins was detected by immunoblotting with an anti-phosphotyrosine monoclonal antibody PY20 (Transduction Laboratories). The intensity of bands was assessed by laser densitometry scanning.

P1-3 Kinase Activity. The activity of P1-3 kinase associated with IRS-1 was assayed by standard protocol provided by the manufacturer of the IRS-1 antibody (UBI). In brief, 500 µg of protein lysate were immunoprecipitated with an anti-IRS-1 polyclonal antibody. The IRS-1 immunoprecipitates were incubated in vitro in the presence of 200 µg/ml phosphatidylinositol (Sigma Chemical Co.) and 10 µCi [γ-32P]ATP for 30 min. The products of the kinase reaction were resolved on TLC plates (Eastman Kodak), and the spots corresponding to P1-3 phosphates were identified by autoradiography. The spots were then cut from the plates, and their radioactivity was counted with a beta counter. For each cell line, P1-3 kinase activity obtained in SFM was taken as 100% (control).

Results

Tam Inhibits the Growth of MCF-7 Breast Cancer Cells Overexpressing Either the IGF-IR or IRS-1. We studied whether Tam is able to inhibit the growth of MCF-7 cells with amplified IGF-IR signaling (MCF-7/IGF-IR and MCF-7/IRS-1 cells). The estrogen growth requirements in these cells are abolished or significantly reduced; however, the cells retain expression of the ER (5, 6). The effect of Tam on growth was studied in MCF-7/IGF-IR, clone 12 (expressing 5 × 10^6 sites/cells; 8-fold IGF-IR overexpression over the levels in MCF-7 cells), MCF-7/IGF-IR, clone 15 (3 × 10^6 sites/cells; 50-fold IGF-IR overexpression), and in MCF-7/IRS-1, clone 3 (a 9-fold overexpression of IRS-1 over that in MCF-7 cells); MCF-7 cells were used as a control. The cells were cultured in PRF-SFM for 4 days in the presence of different concentrations of Tam (0.1–100 nM). Tam treatment suppressed growth of all cell lines in a dose-dependent manner (Fig. 1). Specifically, in all cells, 0.1, 1.0, and 10 nM Tam reduced proliferation by at least 12, 34, and 50%, respectively. The extent of Tam-induced growth inhibition in cells cultured for 4 days in PRF-SFM with 20 ng/ml IGF-I was comparable (data not shown). Treatment with 100 nM Tam was always cytotoxic. Consequently, Tam at a concentration of 10 nM was used in all further experiments.

Tam Interferes with IGF-I-induced Tyrosine Phosphorylation of the IGF-IR in MCF-7/IGF-IR Cells. To investigate the effects of Tam on IGF-IR signaling, we assessed tyrosine phosphorylation and protein levels of the IGF-IR in MCF-7 and MCF-7/IGF-IR, clone 15 cells. In cells cultured in PRF-SFM plus IGF-I, the IGF-IR tyrosine phosphorylation was always elevated compared with that in PRF-SFM (Fig. 2). After 4 days of treatment, the effects of Tam on the basal level of IGF-IR tyrosine phosphorylation were minimal (Fig. 2A); specifically, in several experiments either no modification or slight (~15%) up- or down-regulation were noticeable. However, Tam reduced IGF-I-induced tyrosine phosphorylation by 60% in MCF-7 cells and by 30% in MCF-7/IGF-IR cells (Fig. 2B).

The IGF-IR protein levels were not significantly modulated by Tam, as determined by laser densitometry scanning (Fig. 2). Similarly, the levels of the IGF-IR precursor were not affected by the treatment (Fig. 2A).

Inhibition of Cell Growth by Tamoxifen Is Associated with Dephosphorylation of IRS-1. In all tested cell lines, but especially in the clones with amplified IGF-IR signaling (MCF-7/IGF-IR, clones 12 and 15, and in MCF-7/IRS-1 cells), a basal level of IRS-1 tyrosine phosphorylation was evident even after prolonged culture in PRF-SFM, which reflected cellular response to autocrine IGFs, as shown previously (Refs. 5 and 6; Fig. 3, A and B). The addition of 10 nM Tam to PRF-SFM produced a cytostatic effect (Fig. 1), which was accompanied by a marked dephosphorylation of IRS-1 on tyrosine residues in the cells studied. Specifically, the basal level of IRS-1 tyrosine phosphorylation was reduced by 29, 35, and 48% in MCF-7/IGF-IR, clone 12, MCF-7/IGF-IR, clone 15, and MCF-7/IRS-1 cells, respectively (Fig. 3A). In MCF-7 cells, due to a low basal level of IRS-1
phosphorylation, the effect of Tam was not measurable. The interference of Tam with IRS-1 signaling was further studied in MCF-7/IRS-1 cells (Fig. 3). The IRS-1-stimulated and basal levels of IRS-1 phosphorylation were suppressed in the presence of the drug by approximately 43% (Fig. 3B). The dephosphorylation of IRS-1 was accompanied by its dissociation from both p85 subunit of PI-3 kinase and GRB2 (Fig. 3B). Similar effects of Tam on IRS-1 tyrosine phosphorylation (approximately 27% inhibition) were seen in MCF-7/IGF-IR clone 15 cells (data not shown).

In addition, Tam suppressed the activity of IRS-1-associated PI-3 kinase in cells stimulated with IGF-I; the inhibition by 43, 92, and 128% was seen in MCF-7, MCF-7/IGF-IR, and MCF-7/IRS-1, respectively (Fig. 3C). The effects of Tam on PI-3 kinase in cells cultured in PRF-SFM were not measurable.

In several repeat experiments, IRS-1 protein levels were not affected by long-term treatment with Tam (Fig. 3, A and B).

**Tamoxifen Increases Tyrosine Phosphorylation of SHC.** Of note, in all studied cell lines the cytostatic action of Tam was associated with the elevated tyrosine phosphorylation of p52\text{SHC} and p46\text{SHC} (Fig. 4). The activation of p52\text{SHC} was especially prominent; specifically, compared with SHC status in untreated cells, a 34, 110, and 100% augmentation of p52\text{SHC} tyrosine phosphorylation was observed in MCF-7, MCF-7/IGF-IR, and MCF-7/IRS-1 cells, respectively. Moreover, the hyperphosphorylation of p52\text{SHC} was followed by an increased binding to GRB2 (Fig. 4).

In contrast, in all cell lines, a 4-day exposure to IGF-I decreased tyrosine phosphorylation of p52\text{SHC} by approximately 40% compared with that in PRF-SFM and induced dissociation of SHC/GRB2 complexes (Fig. 4).

Tam treatment produced a consistent down-regulation of p52\text{SHC} and p46\text{SHC} levels by approximately 35% in MCF-7/IGF-IR, clone 15 and MCF-7/IRS-1 cells but not in MCF-7 cells (Fig. 4). In contrast, SHC protein expression was not modulated by IGF-I (Fig. 4).

**Effect of Tamoxifen on ERK2.** Because IRS-1 and SHC, via GRB2/SOS, may activate Ras/MAP signaling pathway, we assessed MAP (ERK2) kinase activity in cells exposed to Tam or cultured for 4 days in PRF-SFM in the presence or absence of exogenous IGF-I. We found no differences in ERK2 activity under these conditions, measured in an in vitro assay, using myelin basic protein as a substrate (data not shown).

**Discussion**

Experimental evidence suggests an important role of the IGF-R in the pathobiology of breast tumors (1–3). Activation of the IGF-IR promotes proliferation and transformation as well as cell-cell and cell-substrate interactions in breast cancer cells (1, 5, 6, 21). Conversely, the blockade of IGF signaling results in the inhibition of breast cancer growth (1, 5, 8). Tam, or its derivative 4-OH-Tam, have been shown to inhibit IGF-IR-dependent growth through different mechanisms, such as down-regulation of autocrine IGF secretion (16) or modulation of IGFBPs expression (1). In addition, in MCF-7 breast cancer cells, Tam and 4-OH-Tam decreased expression of IGF-I binding sites by approximately 30% (19) and 60% (18), respectively.

The effects of Tam on the IGF signal transduction pathway are unknown. Here, we report for the first time the modulation of the IGF-IR intracellular signaling pathways associated with the cytostatic action of Tam. Our studies focused on tyrosine kinase activity and protein levels of the IGF-IR and its two major cellular substrates, IRS-1 and SHC. Preliminary data from Rocha et al. (4) documented that IRS-1 is expressed in primary breast tumors and its levels correlate with increased recurrence. The status of SHC and its relation with
The cells were incubated in PRF-SFM (SFM), PRF-SFM plus 50 ng/ml IGF-I (JGF-I), or PRF-SFM plus 50 ng/ml IGF-I and 10 nM Tam (IGF-LPI'am) for 3 days. IRS-I was precipitated and associated with IRS were determined in original nitrocellulose filters after stripping and reprobing with specific antibodies. C, Tam effects on IRS-I associated P1-3 kinase activity. PRF-SFM (SFM), PRF-SFM plus 10 nM Tam (Tam), or PRF-SFM plus 50 ng/ml IGF-I (IGF-I) for 3 days. IRS-I was precipitated from 300 μg of protein lysates, and tyrosine phosphorylation levels were detected with PY-20 antibody. IRS-I protein level was determined in the original blot, after stripping and reprobing with an anti-IRS-I antibody. Representative results from five experiments are shown.

B, effects of Tam on IRS-I signaling in MCF-7/IRS-I cells. MCF-7/IRS-I, clone 3, was cultured for 4 days in either PRF-SFM (SFM), PRF-SFM plus 50 ng/ml IGF-I alone (IGF-I), PRF-SFM plus 50 ng/ml IGF-I with 10 nM Tam (IGF-I/Tam), or PRF-SFM plus 10 nM Tam (Tam). IRS-I was immunoprecipitated from 250 μg of lysates, and IRS-1 protein level and tyrosine phosphorylation were detected as described above. Amounts of p85 of PI-3 kinase and GRB2 associated with IRS were determined in original nitrocellulose filters after stripping and reprobing with specific antibodies. C, Tam effects on IRS-I associated PI-3 kinase activity. The cells were incubated in PRF-SFM (SFM), PRF-SFM plus 50 ng/ml IGF-I (IGF-I), or PRF-SFM plus 50 ng/ml IGF-I and 10 nM Tam (IGF-I/Tam) for 3 days. IRS-I was precipitated from 500 μg of cell lysates from each cell line. The activity of PI-3 kinase associated with IRS-I was assessed in vitro as described in "Materials and Methods." The results are expressed as percentage of increase over control levels in SFM (100%). Representative data are shown.

Fig. 3. Effects of Tam on IRS-1-mediated signaling. A, effects of Tam on IRS-1 tyrosine phosphorylation. MCF-7 cells, MCF-7/IGF-IR, clones 12 and 15, and MCF-7/IRS-1, clone 3, were incubated in PRF-SFM (SFM), PRF-SFM plus 10 nM Tam (Tam), or PRF-SFM plus 50 ng/ml IGF-I (IGF-I) for 3 days. IRS-I was immunoprecipitated from 300 μg of protein lysates, and tyrosine phosphorylation levels were detected with PY-20 antibody. IRS-I protein level was determined in the original blot, after stripping and reprobing with an anti-IRS-I antibody. Representative results from five experiments are shown.

The attenuation of IRS-I tyrosine phosphorylation by Tam treatment resulted in the persisting dephosphorylation of IRS-I on tyrosine residues, apparently in the presence of both autocrine and exogenous IGF-I. The attenuation of IRS-I tyrosine phosphorylation by Tam was accompanied by down-regulation of IRS-I-associated PI-3 kinase activity and dissociation of GRB2 form IRS-I. Our findings agree with preliminary data of Kleinman et al. (23), who demonstrated that Tam inhibited tyrosine phosphorylation of a Mr 185,000 protein (possibly IRS-1) in MCF-7 cells.

The effect of Tam on SHC was evidently different from that seen in MCF-7/IGF-IR cells, where Tam treatment resulted in the persisting dephosphorylation of IRS-I and SHC. This observation, however, suggests that continuing dephosphorylation of the IGF-IR is not critical for Tam-induced growth arrest.

**Different prognostic markers is not known.** Our experiments with MCF-7 cells expressing antisense RNA to either IRS-1 or SHC demonstrated that normal levels of both substrates are critical in sustaining monolayer and anchorage-independent growth. In addition, IRS-1 signaling appears to play a role in the protection from apoptosis in vitro.
cells were grown in either PRF-SFM (SFM), PRF-SFM plus 10 nM Tam (Tam), or PRF-SFM plus 50 ng/ml IGF-I (IGF-I) for 3 days. SHC protein were immunoprecipitated from 500 μg of cell lysates with an anti-SHC polyclonal antibody followed by detection of tyrosine phosphorylation of SHC with IP:aSHC pAb. The SHC protein and SHC-associated GRB2 were detected in original filters, upon stripping and re-probing with specific antibodies. Representative results of five experiments are shown.

Fig. 4. Effects of Tam on SHC signaling. The cells were grown in either PRF-SFM (SFM), PRF-SFM plus 10 nM Tam (Tam), or PRF-SFM plus 50 ng/ml IGF-I (IGF-I) for 3 days. SHC protein were immunoprecipitated from 500 μg of cell lysates with an anti-SHC polyclonal antibody followed by detection of tyrosine phosphorylation of SHC with IP:aSHC pAb. The SHC protein and SHC-associated GRB2 were detected in original filters, upon stripping and re-probing with specific antibodies. Representative results of five experiments are shown.

for IRS-1. Here, growth inhibition was associated with elevated tyrosine phosphorylation of SHC proteins, especially p52^{SHC}, without up-regulation of SHC protein levels. Importantly, long-term treatment with IGF-I, which promoted growth, concomitantly reduced SHC phosphorylation. Whether up-regulation of SHC phosphorylation is a universal feature of growth arrest or it only represents a characteristic of Tam action is presently unclear. In MCF-7 cells, treatment with genistein or herbimicin inhibited proliferation, which was associated with a reduction of SHC tyrosine phosphorylation observed after 30 min treatment (24). Longer effects of these tyrosine kinase inhibitors were not studied. In our system, higher phosphorylation of SHC in Tam-treated cells was accompanied by GRB2 binding to SHC; however, activation of ERK2 was not observed. Possibly, under Tam treatment, activation of ERK2 via SHC was counteracted by deactivation of this pathway due to disruption of IRS-1 signaling. Alternatively, as suggested by others (24, 25), the ERK2 pathway is not critical in IGF-stimulated growth in MCF-7 cells; thus, it is not a target for Tam action.

In summary, these results demonstrate that Tam differentially modulates IGF-IR signaling in breast cancer cells. The cytostatic effect of Tam is mediated by a continuing inhibition of IRS-1/PI-3 kinase pathway. On the other hand, Tam increases tyrosine phosphorylation of SHC and SHC/GRB2 binding. The biological consequences of the latter effects are presently unknown.

One possible target of Tam action is the tyrosine phosphatase system. Indeed, Freiss and Vignon (26) have recently shown that 4-hydroxymethoxifen up-regulates protein tyrosine phosphatase activity in breast cancer cells. We speculate that Tam activates, most probably through an indirect mechanism, a specific tyrosine phosphatase(s) acting upon IRS-1. On the other hand, Tam may also inhibit tyrosine phosphatase(s) that would specifically affect SHC and/or the IGF-IR. Future experiments with Tam and pure antiestrogens will further explore this issue, especially in relation with such phenomenon as antiestrogen resistance or Tam-induced growth.

References

4. Roche, R. L., Hilsenbeck, S. G., Jackson, J. G., and Yee, D. Insulin-like growth factor binding protein-3 (IGFBP3) and insulin receptor substrate (IRS-1) in primary breast cancer: larger tumors have higher IRS levels and higher levels of IRS-1 are associated with lower disease-free survival (DFS) rate. Breast Cancer Res. Treat. (Suppl.): 58, 1995.

Tamoxifen Interferes with the Insulin-like Growth Factor I Receptor (IGF-IR) Signaling Pathway in Breast Cancer Cells

Marina A. Guvakova and Ewa Surmacz


Updated version Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/57/13/2606

E-mail alerts Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.