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Regulation of Growth Hormone Signaling by Selective Estrogen Receptor Modulators Occurs through Suppression of Protein Tyrosine Phosphatases

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Activation of the Janus kinase 2 (JAK2)/signal transducer and activator of transcription 5 (STAT5) pathway by GH is terminated by the suppressors of cytokine signaling (SOCSs) and protein tyrosine phosphatases, Src homology 2 domain-containing protein tyrosine phosphatase (SHP)-1 and SHP-2. Based on our recent report that estrogen inhibits GH signaling by stimulating SOCS-2 expression, we investigated the effects of selective estrogen receptor modulators (SERMs) on GH signaling in human embryonic kidney (HEK293) and breast cancer (MDA-MB-231) cells expressing human GH receptor and estrogen receptor-α. 17β-Estradiol (E_2) suppressed GH activation of a STAT5-responsive luciferase reporter and JAK2 phosphorylation in both cell models. 4-Hydroxytamoxifen and raloxifene augmented these actions of GH in HEK293 cells but not breast cancer cells. SOCS-2 expression in both cell types was stimulated by E_2 but unaffected by SERMs. In HEK293 cells, SHP-1 was inhibited by raloxifene and 4-hydroxytamoxifen, whereas the latter additionally inhibited SHP-2. The phosphatases were unaffected by E_2. In breast cancer cells, phosphatase activity was not altered by SERMs or E_2. In summary, estrogen inhibited the JAK2/STAT5 signaling of GH and stimulated SOCS-2 expression in both HEK293 and breast cancer cells. By contrast, SERMs augmented GH signaling by reducing SHP activities in HEK293 cells and had no effect on both in breast cancer cells. We provide the first evidence for a novel mechanism regulating GH signaling, in which SERMs enhance GH activation of the JAK2/STAT5 pathway in a cell-type-dependent manner by attenuating protein tyrosine phosphatase activities. (*Endocrinology* 148: 2417–2423, 2007)

GH EXERTS ITS BIOLOGICAL actions by acting through specific receptors (GHRs) on the cell surface (1, 2). Association of GH with GHRs leads to activation and phosphorylation of Janus kinase 2 (JAK2), which phosphorylates the signal transducer and activator of transcription 5 (STAT5). STAT5 induces expression of GH-responsive genes, including IGF-I (3, 4). GH signaling is terminated by two mechanisms, one involving the suppressors of cytokine signaling (SOCSs) and the other, the protein tyrosine phosphatases (PTPs) (1). GH induces the expression of SOCS-1, -2, and -3, which feed back to suppress activation of the JAK2/STAT5 cascade (5). The Src homology 2 domain-containing PTPs, SHP-1 and SHP-2, inactivate GH signaling by dephosphorylating GHR, JAK2, and STAT5 (6–8).

Estrogens are important regulators of GH action (1). Oral administration of estrogen reduces IGF-I levels in blood and impairs the metabolic action of GH (9–12). Selective estrogen receptor modulators (SERMs), as exemplified by tamoxifen and raloxifene, are nonsteroidal ligands of estrogen receptors (ERs), which exhibit tissue-dependent estrogen agonistic and antagonistic actions (13, 14). Tamoxifen and raloxifene antagonize estrogen action in breast tissue, display estrogen-like action in bone, but exert diverse effects in the uterus. SERMs reduce serum IGF-I levels in normal subjects and patients with breast cancer or acromegaly (15–20), suggesting that, like estrogens, they attenuate the effect of GH. Tamoxifen reduces pituitary GH secretion (21–23) and hepatic GHR expression (24), providing potential mechanisms for their effects on the GH/IGF-I system.

The effects of SERMs on GH signaling have not been investigated. Recently we reported that estrogen inhibits GH action by suppressing JAK2 phosphorylation (25). The inhibitory action is exerted through stimulation of SOCS-2 expression. In the present study, we examined the effects of tamoxifen and raloxifene on GH signaling in human embryonic kidney (HEK293) and breast cancer (MDA-MB-231 and T47D) cells.

Materials and Methods

Reagents and plasmids

Recombinant human GH was produced in-house (26). Raloxifene (Ral) was kindly provided by Colin Watts (Cancer Research Program, Garvan Institute of Medical Research, Sydney, Australia). Other reagents were purchased from various suppliers as follows: cell culture reagents and TRIzol reagent from Life Technologies, Inc. (Melbourne, Australia); the antiestrogen, ICI182,780, from ICI Pharmaceuticals (Macclesfield, UK); 17β-estradiol (E_2), 4-hydroxytamoxifen (4HT), dexamethasone (Dex), actinomycin D, and sodium orthovanadate from Sigma (St. Louis, MO); Omniscript RT kit and nonliposomal (Effectene)

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Abbreviations: Dex, Dexamethasone; E_2, 17β-estradiol; ER, estrogen receptor; GHR, GH receptor; 4HT, 4-hydroxytamoxifen; JAK, Janus kinase; PTP, protein tyrosine phosphatase; Ral, raloxifene; SERM, selective estrogen receptor modulator; SHP, Src homology 2 domain-containing PTP; SOCS, suppressor of cytokine signaling; STAT, signal transducer and activator of transcription.

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transfection reagent from QIAGEN (Clifton Hill, Victoria, Australia); rabbit polyclonal antibodies against JAK2 (HR-758 and C-12), SHP-1 (C-19), and SHP-2 (N-16) from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); antiphosphotyrosine monoclonal antibody (4G10) and PTP Assay Kit 1 from Upstate Biotechnology Inc. (Lake Placid, NY); ImmunoPure Protein A/G agarose gel and bicinchoninic acid protein assay kit from Pierce Chemical Co. (Rockford, IL); Complete protease inhibitor cocktail (with inhibitors for serine, cysteine, metalloproteases, and calpains) and LightCycler-Fast Start Reaction Mix SYBR Green I from Roche Molecular Biochemicals (Sydney, Australia).

Expression plasmids for human GHR (pcDNAI/Amp-GHRfl), human ERα (pCMV-ERgly-neo), and β-galactosidase (IEP-βgal-CMV) were generous gifts from Richard Ross (Sheffield University, Sheffield, UK), Craig Jordan (Robert H. Lurie Cancer Centre, Chicago, IL), and Gerald Clesham (University of Cambridge, Cambridge, UK), respectively. Luciferase reporter constructs containing six copies of a synthetic STAT5-responsive element fused to a minimal thymidine kinase promoter (pUC18-LHRE/TK) (27) or a *Nde*I-*Xho*I fragment of the rat β-casein promoter (nt −344 to −1) in a pLucDSS plasmid (pZZ1) (28) were kindly provided by Paul Kelly (Faculty of Medicine Necker, Paris, France) and Bernd Groner (Institute of Experimental Cancer Research, Freiburg, Germany), respectively. PCR primers for human SOCS-2 (forward: 5′-GGATGGTACTGGGGAAGTATGACTG-3′; reverse: 5′-AGTCGATCAGATGAACCACACTGTC-3′) (25) were obtained from Sigma Genosys (Sydney, Australia).

Luciferase reporter assay

A STAT5 reporter assay was performed in HEK293 cells stably expressing GHR (293GHR) (29), MDA-MB-231, and T47D cells as described (25) using luciferase reporter constructs with the STAT5-responsive element or the rat β-casein promoter. Briefly, after being precultured in phenol red-free medium supplemented with 10% charcoal-stripped fetal calf serum for 3 d, the cells were transiently transfected overnight with the reporter constructs (0.1 μg), expression plasmids for human ERα (0.1 μg) and β-galactosidase (0.01 μg), using the Effectene reagent. Human GHR was coexpressed in MDA-MB-231 cells to render the cells GH responsive, whereas no transfection with ER and GHR was needed in T47D cells (25). The cells were then treated in triplicate with 500 ng/ml GH, 250 nM Dex (to augment GH activation of the STAT5 reporter) (25, 30), and varying concentrations of E_2, 4HT, or Ral at 37 C for 6 or 18 h before measurement of luciferase and β-galactosidase activity. All experiments were performed in phenol red-free medium under serum-free conditions.

Western analysis of JAK2 and PTP

The effects of E_2 and SERMs on GH-induced JAK2 phosphorylation in 293GHR and breast cancer cells expressing ERα were examined by Western blotting analysis as previously described (25). Cells were pretreated with 100 nM E_2, 4HT, or Ral at 37 C for 3 h and then with 500 ng/ml GH for 2 or 15 min. Cell lysates were immunoprecipitated with the anti-JAK2 polyclonal antibody (HR-758), resolved by SDS-PAGE and transferred onto a polyvinylidene fluoride membrane. The membrane was immunoblotted with the antiphosphotyrosine antibody (4G10) and then stripped and reprobed with the anti-JAK2 antibody (C-12). The bands were visualized by chemiluminescence and quantified by densitometry. Relative abundance of phosphorylated to total JAK2 was recorded and presented as percentages of control treated with GH alone.

The abundance of SHP-1 and SHP-2 protein in 293GHR cells was assessed by Western blotting analysis. After treatment with 100 nM E_2 or SERMs at 37 C for 2 or 6 h, 50 μg of whole-cell lysates were resolved by SDS-PAGE on 10% gel and blotted onto a polyvinylidene fluoride membrane. The membrane was treated with blocking buffer [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5% skim milk powder, and 0.1% Tween 20] and probed with 5 μg per 5 ml of antibodies against SHP-1 or SHP-2. The signal was developed with donkey antirabbit Ig-horseradish peroxidase by chemiluminescence.

Quantitative PCR of SOCS-2

The effects of E_2 and SERMs on SOCS-2 expression in 293GHR and breast cancer cells expressing ERα were examined by reverse transcription accompanied by quantitative PCR as described (25). Cells were treated in triplicate with 100 nM E_2, 4HT, or Ral as well as 500 ng/ml GH in the cotreatment studies of SERMs and GH for 0.5, 1, 2, and 4 h. Total RNA was extracted using TRIzol reagent, reverse transcribed, and quantified by real-time PCR with LightCycler-Fast Start Reaction Mix SYBR Green I in a LightCycler (Roche). The amplification program included an initial denaturation step at 95 C for 10 min, followed by 40 cycles of denaturation at 95 C for 0.5 sec, annealing at 56 C for 5 sec, and extension at 72 C for 10 sec, with ramping rates at 20 C/sec. Values of crossing point (the first turning point) for standards were used to construct a calibration curve, from which copy numbers for the samples were estimated. All samples were assayed at the same time for statistical comparison.

PTP assay

Activities of SHP-1 and SHP-2 were measured by immunoprecipitating the phosphatases with respective antibodies, incubating the precipitates with a synthetic phosphopeptide (NH_2-RRLIEDADpYAARG-COOH) (Upstate PTP Assay Kit 1) as substrate and quantifying free phosphate produced by the Malachite Green colorimetric method (31), as recommended by the manufacturer. Accordingly, 293GHR and breast cancer cells were treated in triplicate with 100 nM SERMs or E_2 at 37 C for 2, 4, and 6 h and solubilized in lysis buffer [50 mM Tris (pH 7.2), 0.14 M NaCl and 0.4% Triton X-100] with Complete protease inhibitor cocktail. Lysates (5 mg) were incubated with 2 μg of antibodies against SHP-1 or SHP-2 and precipitated with Protein A/G agarose gel. After two washes with lysis buffer, the samples were preincubated in 45 μl assay buffer [25 mM HEPES (pH 7.2), 50 mM NaCl, 5 mM dithiothreitol, and 2.5 mM EDTA] at 37 C for 15 min, followed by addition of 5 μl 1 mM phosphopeptide and further incubation for 30 min. The reaction was terminated by addition of 100 μl Malachite Green solution, and color was allowed to develop at 25 C for 5 min. A_{620nm} was measured in a microplate reader and compared with a standard curve of 2 to 6 μM free phosphate. Protein content of the lysates was measured using the Bicinchoninic acid protein assay. The PTP activity was expressed as pmol phosphate per mg lysate protein per ml (picomoles per milligram per milliliter).

Statistical analysis

All experiments were repeated three times unless stated otherwise. Mean ± SEM of results from multiple experiments of the same study are reported. Data were analyzed by paired *t* test or ANOVA (StatView 4.5; Abacus Concepts Inc., Berkeley, CA) where appropriate, and significance was set at $P < 0.05$.

Results

SERM effects on estrogen regulation of GH signaling

We first compared the effects of SERMs on the E_2 regulation of GH action in HEK293 and breast cancer cells. GH stimulated STAT5 reporter activity by 8.8 ± 0.8-fold ($P < 0.001$) and 5.5 ± 1.1-fold ($P < 0.005$) in HEK293 cells stably expressing GHR (293GHR) and in breast cancer (MDA-MB-231) cells, respectively. E_2 significantly reduced GH-induced reporter activity in the two cell lines by 44 ± 2 and 40 ± 5%, respectively ($P < 0.05$; Fig. 1). Addition of excess (1 μM) of 4HT, Ral, and the antiestrogen, ICI182,780, markedly attenuated the inhibitory effect of E_2. Interestingly, 4HT and Ral alone significantly augmented GH-induced reporter activity in 293GHR but not breast cancer cells, suggesting that in addition to counteracting the estrogen action, SERMs exerted cell-type dependent, enhancing effects on GH signaling.

JAK2/STAT5 signaling in 293GHR cells

To investigate the regulation of GH signaling by SERMs in 293GHR cells, effects on GH activation of the STAT5 reporter and a luciferase reporter containing the β-casein promoter,

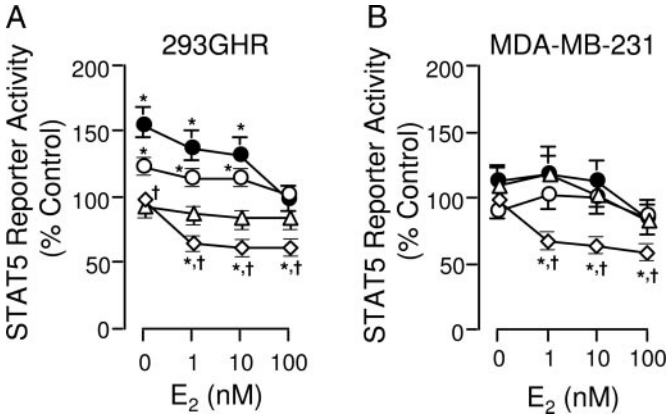

FIG. 1. Effects of SERMs and antiestrogen on E_2 regulation of GH-induced STAT5 transcriptional activity. 293GHR (A) and breast cancer (MDA-MB-231) (B) cells were transiently transfected with an ERα expression plasmid, a luciferase reporter containing the STAT5-responsive elements and in the case of breast cancer cells, with a GHR expression plasmid. The cells were then treated with 500 ng/ml GH, 250 nM Dex, increasing concentrations of E_2 alone (*diamonds*) and with 1 μM of 4HT (*closed circles*), Ral (*open circles*), or ICI182,780 (*triangles*) for 18 h before luciferase activity measurement. The mean reporter activity ($\pm$SEM) was expressed as percentage of samples treated with GH only. *, $P < 0.05$ *vs.* GH-treated control; †, $P < 0.05$ *vs.* GH plus SERMs or ICI182,780.

a native STAT5-responsive promoter, were examined. In contrast to E_2, 4HT and Ral augmented GH-induced STAT5 reporter activity in a dose-dependent manner, to a maximum of 188 $\pm$ 11% ($P < 0.001$) and 170 $\pm$ 18% of control ($P < 0.05$), respectively (Fig. 2A). Similarly, 4HT and Ral increased the GH-stimulated β-casein reporter activity to 145 $\pm$ 8% and 148 $\pm$ 11%, respectively, whereas E_2 decreased the response to 51 $\pm$ 3% of control ($P < 0.05$, Fig. 2B). As Dex was added to enhance the STAT5 reporter activation by GH (25, 30), its effects on the responses to E_2 and SERMs were examined. As shown in Fig. 2C, E_2 reduced and SERMs increased the GH-induced reporter activity to the same levels with and without Dex. GH stimulation of JAK2 phosphorylation was suppressed by E_2 to 57 $\pm$ 4% ($P = 0.01$; Fig. 2D) and enhanced by SERMs to 177 $\pm$ 15 and 184 $\pm$ 11% ($P < 0.05$), respectively. Thus, E_2 and SERMs exerted completely opposite effects on GH activation of the JAK2/STAT5 pathway.

The role of ERα in mediating the effects of SERMs on GH signaling was examined using ICI182,780. As shown in Fig. 3, addition of 1 μM of the antiestrogen completely abrogated the modulatory actions of E_2, 4HT, and Ral on GH activation of the STAT5 reporter.

Actinomycin D

To investigate whether *de novo* gene expression was required for SERM enhancement of GH signaling, the effects of a transcription inhibitor, actinomycin D, were examined. In the absence of actinomycin D, 4HT and Ral increased JAK2 phosphorylation to 158 $\pm$ 8 and 187 $\pm$ 4% of GH-treated control, respectively ($P < 0.05$; Fig. 4). Pretreatment with the inhibitor completely abolished the effects of SERMs (101 $\pm$ 10 and 100 $\pm$ 15%, respectively), suggesting that the enhancing effects of SERMs were secondary to a transcriptional response.

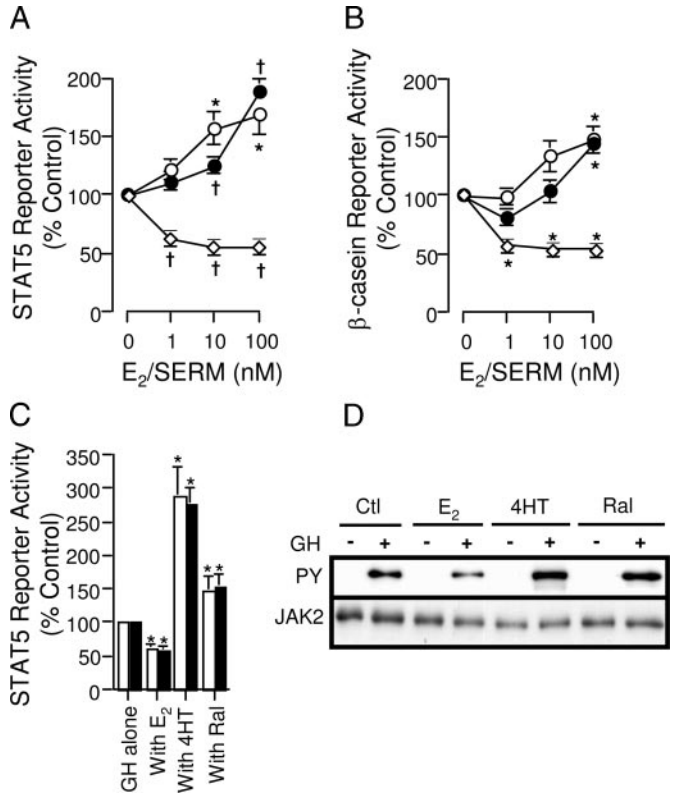

FIG. 2. Effects of E_2 and SERMs on GH-induced STAT5 transcriptional activity and JAK2 phosphorylation. 293GHR cells were transiently transfected with an ERα expression plasmid and a luciferase reporter containing the STAT5-responsive elements (A) or a β-casein promoter reporter and a JAK2 expression plasmid (B). The cells were then treated with 500 ng/ml GH, 250 nM Dex, and E_2 (*diamonds*), 4HT (*closed circles*), or Ral (*open circles*) at indicated concentrations for 6 h (A) or 18 h (B) before luciferase activity measurement. The mean reporter activity ($\pm$SEM) was expressed as percentage of GH-treated control. *, $P < 0.05$; †, $P < 0.01$ *vs.* GH-treated control. C, 293GHR cells transfected with the STAT5 reporter were treated with 500 ng/ml GH, 100 nM E_2, or SERMs and without (*white bars*) or with (*black bars*) 250 nM Dex. *, $P < 0.05$ *vs.* respective GH alone control. D, Representative Western blots of phosphorylated (*top panel*) and total JAK2 (*bottom panel*) in cells treated with GH, E_2, 4HT, and/or Ral as described in *Materials and Methods*. Ctl, Vehicle control; PY, tyrosine phosphorylation.

SOCS-2 expression

Because estrogen inhibition of GH signaling is mediated by SOCS-2 (25), the effects of SERMs on SOCS expression in 293GHR cells were examined. Whereas E_2 increased SOCS-2 mRNA abundance to 178 $\pm$ 27% of control ($P < 0.05$), neither 4HT nor Ral had a significant effect (Fig. 5A). Because GH stimulates SOCS expression to terminate its own signaling, the effects of SERMs on GH induction of SOCS-2 were next studied. As shown in Fig. 5B, GH increased SOCS-2 mRNA abundance by more than 3-fold. The magnitude of response was unaffected by cotreatment with 4HT or Ral. Taken together, SERMs did not regulate basal or GH-stimulated SOCS-2 expression.

PTP activity

The possible involvement of PTPs in SERM regulation of GH signaling in 293GHR cells was examined using vanadate,

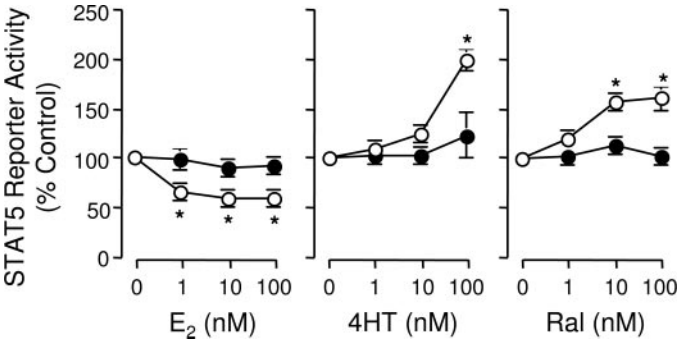

FIG. 3. Effects of antiestrogen on the actions of E_2 and SERMs. 293GHR cells transfected with an ERα expression plasmid and the STAT5 reporter were treated without (*open circles*) or with (*closed circles*) 1 μM ICI182,780 for 30 min and then with 500 ng/ml GH, 250 nM Dex, and 100 nM E_2, 4HT, or Ral for 6 h before the STAT5 reporter activity was measured. Results are expressed as mean ± SEM. *, $P <$ 0.05 *vs.* ICI-treated samples at corresponding concentrations.

a phosphatase inhibitor (32, 33). Cotreatment with vanadate significantly increased GH stimulation of STAT5 reporter activity from 3.6 ± 0.7- to 5.2 ± 0.7-fold ($P < 0.05$; Fig. 6A). E_2 reduced the GH response to 62 ± 4% of control, and addition of vanadate did not affect the E_2 attenuation (65 ± 3%). 4HT and Ral enhanced GH-induced reporter activity to levels similar to that with vanadate. Cotreatment with SERMs and vanadate did not further increase the reporter responses.

To examine whether SERMs and vanadate exerted additive effects on the transcriptional activity of GH, cotreatment with these reagents at submaximal concentrations of SERMs (25 nM) and vanadate (1 μM) was studied. At these concentrations, 4HT and Ral alone enhanced GH-induced reporter activity to 135 ± 7 and 115 ± 5% of GH-treated control, respectively ($P < 0.05$; Fig. 6B). Vanadate increased the GH response to 116 ± 5% ($P < 0.05$ *vs.* GH-treated control) and further amplified the effects of 4HT and Ral to 149 ± 8 and 126 ± 3%, respectively ($P < 0.05$ *vs.* SERM alone). Taken together, these findings indicate that PTPs may be involved in the augmentation of GH signaling by SERMs.

To investigate which of the PTPs might be involved in the SERM regulation, the activities of SHP-1 and SHP-2, which are known to affect GH signaling, were measured. The basal activities of SHP-1 and SHP-2 were 41 ± 6 and 48 ± 8 pmol/mg·ml, respectively. 4HT and Ral reduced SHP-1 activity in a time-dependent manner to 66 ± 6 and 58 ± 9% of control, respectively ($P < 0.05$), whereas no effect was observed with E_2 (Fig. 7A). 4HT also reduced SHP-2 activity to 61 ± 6% ($P < 0.05$), whereas Ral and E_2 did not.

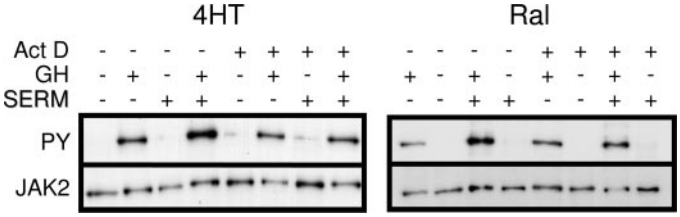

FIG. 4. Effects of transcription inhibitor on the actions of SERMs. Representative Western blots of phosphorylated (*top panel*) and total (*bottom panel*) JAK2 in 293GHR cells transfected with an ERα expression plasmid and treated with 5 μg/ml actinomycin D (Act D) for 1 h, followed by treatment with 100 nM 4HT or Ral for 3 h and with 500 ng/ml GH for 2 min, as indicated.

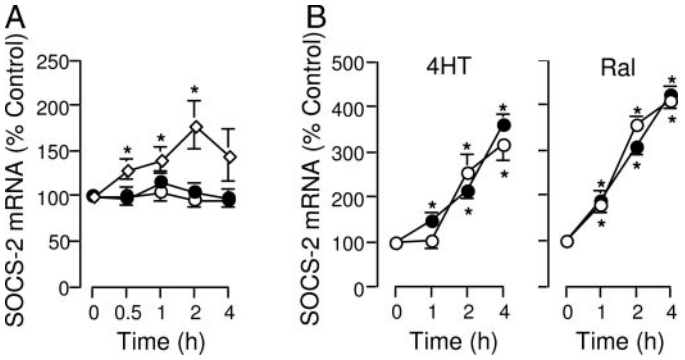

FIG. 5. Effects of E_2 and SERMs on SOCS-2 mRNA expression. 293GHR cells expressing ERα were treated with 100 nM E_2 (*diamonds*), 4HT (*closed circles*), or Ral (*open circles*) (A) and with 500 ng/ml GH alone (*open circles*) or together with 100 nM 4HT or Ral (*closed circles*) (B) for indicated time, followed by quantitation of SOCS-2 mRNA by RT-PCR as described in *Materials and Methods*. Results are expressed as mean ± SEM. *, $P < 0.05$ *vs.* 0 h.

To determine whether the decline in SHP-1 and SHP-2 activities might result from changes in protein content, their abundance was examined by Western blotting analysis. As shown in Fig. 7B, E_2, 4HT, and Ral did not alter protein content of SHP-1 or SHP-2. The collective data indicate that decrease in SHP-1 and SHP-2 activities was unlikely to be caused by reduced expression.

JAK2/STAT5 signaling in breast cancer cells

To investigate whether E_2 and SERMs affected GH signaling, SOCS-2 expression, and PTP activity in another cell type, their effects were examined in breast cancer (MDA-MB-231 and T47D) cells. GH significantly stimulated STAT5 reporter activity by 5.5 ± 1.1- and 2.4 ± 0.2-fold ($P < 0.05$),

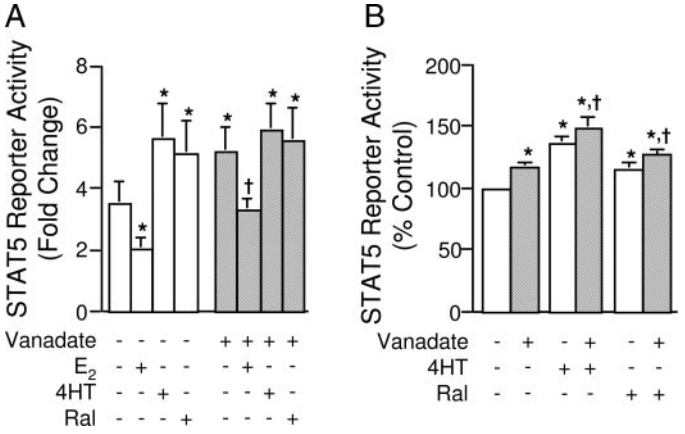

FIG. 6. Effects of phosphatase inhibitor on the actions of E_2 and SERMs. A, 293GHR cells expressing ERα were treated without (*open bars*) or with 1 mM vanadate (*hatched bars*) for 1 h and then with 500 ng/ml GH, 250 nM Dex, and 100 nM E_2, 4HT, or Ral for 18 h as indicated before the STAT5 reporter activity was measured. Results are expressed as mean ± SEM. *, $P < 0.05$ *vs.* GH; †, $P < 0.05$ *vs.* GH plus vanadate. B, The cells were treated with 500 ng/ml GH, 250 nM Dex, and submaximal concentrations of vanadate (1 μM) and/or SERMs (25 nM) as indicated. *, $P < 0.05$ *vs.* GH; †, $P < 0.05$ *vs.* corresponding samples treated with SERM and not vanadate.

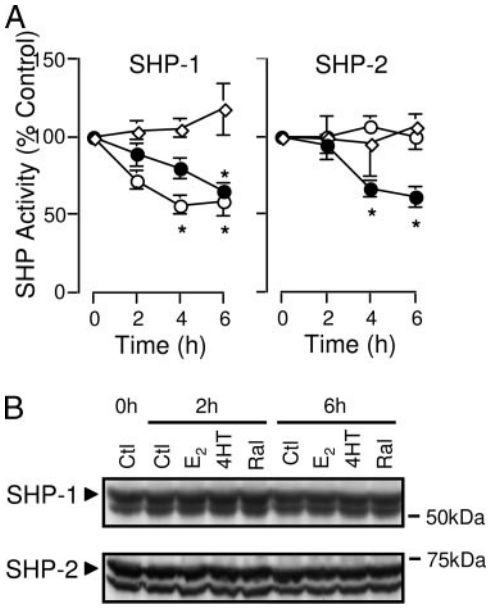

FIG. 7. Effects of E_2 and SERMs on PTPs. A, 293GHR cells expressing ERα were treated with 100 nM E_2 (*diamonds*), 4HT (*closed circles*), or Ral (*open circles*) for time as indicated, followed by measurement of SHP-1 (*left panel*) and SHP-2 activity (*right panel*) as described in *Materials and Methods*. Results are expressed as mean ± SEM. *, $P < 0.05$ *vs.* 0 h. B, A representative Western blot of SHP-1 (*top panel*) and SHP-2 (*bottom panel*) in cells treated with vehicle control (Ctl), 100 nM E_2, 4HT, or Ral for 2 or 6 h.

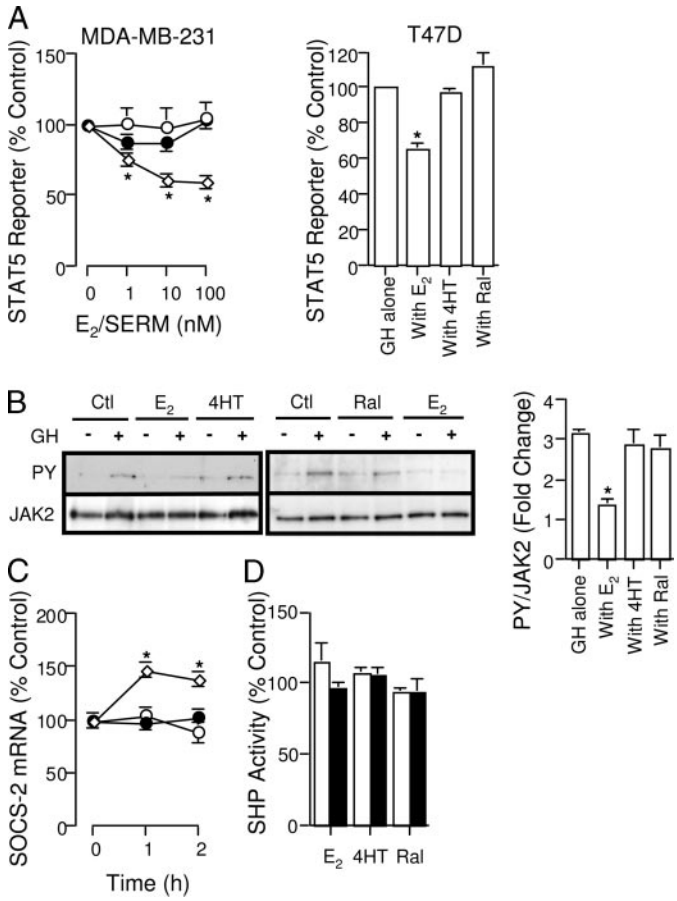

FIG. 8. Breast cancer cells. A, MDA-MB-231 cells were treated with 500 ng/ml GH, 250 nM Dex and E_2 (*diamonds*), 4HT (*closed circles*), or Ral (*open circles*) at indicated concentrations for 18 h before measurement of the STAT5 reporter activity. T47D cells were treated with GH, Dex, and E_2 or SERMs at 100 nM. Results are expressed as mean ± SEM. *, $P < 0.05$ *vs.* GH. B, Representative Western blots of phosphorylated (*top panel*) and total JAK2 (*bottom panel*) in MDA-MB-231 cells transiently expressing GHR and JAK2 and treated with GH, E_2, and/or SERMs as indicated. The bands were quantified by densitometry and plotted as mean fold change (±SEM) of untreated control (Ctl). *, $P < 0.05$ *vs.* GH alone. C, RT-PCR of SOCS-2 mRNA in cells treated with 100 nM E_2 (*diamond*), 4HT (*closed circles*), or Ral (*open circles*) for 1 and 2 h. *, $P < 0.01$ *vs.* 0 h. D, Activities of SHP-1 (*white bars*) and SHP-2 (*black bars*) in cells treated with 100 nM E_2, 4HT, or Ral for 6 h. PY, Tyrosine phosphorylation.

respectively, and the effect was reduced by E_2 to 60 ± 5 and 65 ± 4% of GH-treated control, respectively ($P < 0.05$; Fig. 8A). In contrast to 293GHR cells, 4HT and Ral did not significantly affect GH-induced reporter activity in these breast cancer cells. E_2 also reduced GH stimulation of JAK2 phosphorylation in MDA-MB-231 cells from 3.1 ± 0.1- to 1.4 ± 0.1-fold of untreated control ($P < 0.01$; Fig. 8B), whereas 4HT and Ral had no effect (2.9 ± 0.4- and 2.8 ± 0.3-fold, respectively). Furthermore, E_2 but not SERMs increased SOCS-2 mRNA abundance up to 1.5-fold of control ($P < 0.01$; Fig. 8C). E_2, 4HT, and Ral had no significant effect on the activities of SHP-1 and SHP-2 (Fig. 8D). In summary, E_2 inhibited GH activation of the JAK2/STAT5 pathway and stimulated SOCS-2 expression in breast cancer cells as in 293GHR cells, whereas SERMs did not affect GH signaling, SOCS-2 expression, and PTP activity.

Discussion

We have shown that estrogen suppressed and SERMs enhanced GH activation of the JAK2/STAT5 pathway in HEK293 cells. They acted separately and selectively on two different systems, which negatively regulate GHR function. Estrogen stimulated SOCS-2 expression without affecting PTP activity, whereas SERMs suppressed PTP activity without affecting SOCS-2 expression. In breast cancer cells, estrogen inhibited GH signaling and stimulated SOCS-2 expression as in HEK293 cells. In contrast, SERMs did not affect GH signaling, PTP activity, and SOCS-2 expression in breast cancer cells but blocked the suppressive effect of estrogen on GH activation of the JAK2/STAT5 pathway. The suppression of PTP by SERMs resulting in enhancement of GH signaling has not been previously reported, and the effect appears to be tissue specific.

The JAK2/STAT5 pathway plays a key role in mediating GH action (2, 34), including induction of IGF-I expression (3, 4). The signaling is negatively regulated by members in the SOCS and PTP families through distinct mechanisms (1). SOCSs block activation and promote ubiquitin-mediated degradation of JAK and STAT proteins (5), whereas PTPs terminate signal transduction by dephosphorylating GHR, JAK2, and STAT5 (6–8). Both systems are activated by GH (7, 35), and their regulation provides a potential target of feedback control of GH action.

Estrogen suppressed GH signaling in HEK293 and breast cancer cells through stimulation of SOCS-2 expression, as previously reported (25). The observations in the breast cancer cells are interesting because both GH and estrogen are

stimulators of breast development (36, 37). However, it has recently been reported that estrogen with progesterone effectively inhibits GH activation of the MAPK pathway and tumorigenic development of mammary tissues in spontaneous dwarf rats (38). These findings highlight the physiological significance of the interference of GH signaling by estrogen.

SERMs are a class of estrogenic compounds, which bind ERs and exert estrogen agonistic and antagonistic effects in a tissue-specific manner. In this study, we uncovered a novel estrogen antagonistic property of SERMs in HEK293 cells. Instead of acting as a classical ER antagonist as observed in breast cells, both tamoxifen and raloxifene exerted an enhancing effect that was directly opposite to estrogen. This effect was achieved through a mechanism distinct from that mediating the inhibitory action of estrogen. These findings indicate that SERMs may counteract the estrogen antagonistic action on GH signaling through two different mechanisms: a blocking and an opposing action. In breast cancer cells, SERMs antagonize the inhibitory action of estrogen likely by competing for ER binding as a pure antiestrogen. In contrary, SERMs exhibit intrinsic stimulatory effects on GH signaling in kidney cells.

It has been shown that GH is expressed in some cell lines and enables activation of the GHR function in an autocrine/paracrine manner (39, 40). It is possible that estrogen and SERMs might modulate GH expression in the kidney and breast cancer cells to affect the responses of receptor signaling. However, this possibility is considered unlikely because there was no detectable GH in these cells before and after treatment with estrogen and SERMs (Leung, K.-C., unpublished observations).

The mechanism(s) by which SERMs negatively regulate PTPs are unknown. Possible mechanisms include: 1) inhibiting PTP expression, 2) directly inactivating PTP activity, and 3) regulating expression of a factor(s), which modulates PTP activity. The first mechanism is unlikely because no significant change in the protein abundance of SHP-1 and SHP-2 by SERM treatment was observed. It is known that PTPs can be inactivated by epidermal growth factor, platelet-derived growth factor, and TGFβ1 through protein oxidation (41). These growth factors induce production of reactive oxygen species such as hydrogen peroxide, which oxidize an essential cysteine residue in the catalytic site of phosphatases leading to loss of enzyme activity. However, this is an unlikely mechanism because both tamoxifen and raloxifene suppress the production of reactive oxygen species (42, 43). The results from the actinomycin D studies suggest that the effects of SERMs are indirect and involve transcriptional regulation of a factor(s), which reduces PTP activity. Investigation on the possible existence of such factor(s) is the subject of current studies.

Tamoxifen and raloxifene display estrogen agonistic and antagonistic activities in a tissue-specific manner (13, 14). We show here that SERMs up-regulated GH signaling by suppressing PTP activity in HEK293 cells but not breast cancer cells. These results suggest that cell type-specific factors are likely to contribute to PTP inhibition by SERMs. Tissue specificity of SERM action has been the subject of intensive studies. There is strong evidence indicating that this arises from several mechanisms including differences in cellular content of transcriptional coregulators, ER subtypes (ERα *vs.* ERβ), promoter structures of target genes, and the context of their interaction (44). Whereas the enhancing effect of SERMs was demonstrated here in HEK293 cells, these findings provide the molecular basis for understanding SERM regulation of GH signaling in other tissues.

Although SERMs enhanced GH signaling in HEK293 cells, there was a subtle difference between 4HT and Ral in relation to their action on SHP-1 and SHP-2. Whereas tamoxifen inhibited both PTPs, raloxifene reduced the SHP-1 activity only, indicating that effects in the same tissue are not identical. This observation may be explained by the findings from gene expression studies that tamoxifen and raloxifene do not regulate an identical set of genes (45–47).

In conclusion, estrogen and SERMs exert opposite effects on the JAK2/STAT5 signaling of GH through differential effects on SOCS-2 and PTPs, respectively. The findings of variable GH regulatory effects in different cell types would add a new dimension to the pharmacological properties of SERMs, including the observation that SERMs antagonize the inhibitory effect of estrogen on GH signaling through two mechanisms, the classical antagonist and a novel opposing (stimulatory) action. The significance of the stimulatory effect of SERMs on GH signaling deserves future study.

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