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Human glutathione S-transferase P1-1 functions as an estrogen receptor α signaling modulator

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ABSTRACT

Estrogen receptor α (ERα) plays a crucial role in estrogen-mediated signaling pathways and exerts its action as a nuclear transcription factor. Binding of the ligand-activated ERα to the estrogen response element (ERE) is a central part of ERα-associated signal transduction pathways and its aberrant modulation is associated with many disease conditions. Human glutathione S-transferase P1-1 (GSTP) functions as an enzyme in conjugation reactions in drug metabolism and as a regulator of kinase signaling pathways. It is overexpressed in tumors following chemotherapy and has been associated with a poor prognosis in breast cancer. In this study, a novel regulatory function of GSTP has been proposed in which GSTP modulates ERE-mediated ERα signaling events. Ectopic expression of GSTP was able to induce the ERα and ERE-mediated transcriptional activities in ERα-positive but GSTP-negative MCF7 human breast cancer cells. This inductive effect of GSTP on the ERE-transcription activity was diminished when the cells express a mutated form of the enzyme or are treated with a GSTP-specific chemical inhibitor. It was found that GSTP inhibited the expression of the receptor interacting protein 140 (RIP140), a negative regulator of ERα transcription, at both mRNA and protein levels. Our study suggests a novel non-enzymatic role of GSTP which plays a significant role in regulating the classical ERα signaling pathways via modification of transcription cofactors such as RIP140.

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1. Introduction

Estrogen receptor α (ERα) is a transcription factor crucial for estrogen-responsive gene transcription. The dimers of ligand-bound ERα interact with the specific DNA sequences such as the estrogen response element (ERE; GGTCAcagTGACC), activating protein (AP)-1 binding sequences (ATGAGTCAT), or GC rich specificity protein (Sp) binding sequences ((G/T)GGGCGG(G/A)(G/A)(C/T))-located in the regulatory region of target gene promoters [1]. Interaction of ERα with the specific responsive elements leads to the induction of target gene transcription depending on the cell type and promoter context by recruiting a variety of coregulatory proteins that stabilize the transcription initiation complex or alter chromatin structure.

ERα target genes including cyclin D1, progesterone receptor, and trefoil factor 2 are tightly regulated since these genes are closely implicated in cell proliferation, survival and/or death, thus playing vital roles in development, growth, and tumorigenesis [2]. The classical mechanism of ER action involves binding of ERα to the ERE and is attributed to approximately two thirds of estrogen activated ERα action [3]. Thus, regulation of the classical ERα action is a critical part of in the management of receptor-mediated disease conditions.

Human glutathione S-transferase P1-1 (GSTP; EC 2.5.1.18) catalyzes thioether conjugation of glutathione (GSH) with potentially toxic electrophile reactive intermediates, acting as the detoxifying enzyme [4]. In addition, GSTP has a diversity of regulatory functions such as JNK signaling pathways and Cdk5 kinase activity, and it is therefore involved in cell death and/or survival mechanisms [5,6]. Elevated GSTP expression of has been demonstrated in tumors and believed to play a role in drug resistance. For

Abbreviations: AF, activating function; AP, activating protein; ANOVA, analysis of variance; DMSO, dimethylsulfoxide; ERα, estrogen receptor α; ERE, estrogen response element; GSTP, human glutathione S-transferase P1-1; 4-OHT, 4-hydroxytamoxifen; IgG, immunoglobulin G; PBS, phosphate buffered saline; PVDF, poly vinylidene fluoride; RIP, regulatory interacting protein; SERM, selective estrogen receptor modulators; Sp, specificity protein; TBST, tris-buffered saline with 0.5% Tween-20.

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example, a study showed that GSTP expression indicates chemotherapy resistance and has been associated with a poor prognosis in breast cancers [7].

The recent studies show that the presence of GSTP predicts poor pathological complete response in ERα-negative breast cancer during neoadjuvant chemotherapy [8]. Various studies also indicate that GSTP expression accompanying loss of ERα at the either mRNA or protein level may contribute to poor prognosis in breast cancer and the mechanisms of drug resistance [9,10]. Although the regulation of GSTP at either the gene or protein level via ERα and its role in the development of endocrine therapy resistance remain unclear, GSTP expression is increased with decreased expression of ERα and has been associated with an altered response of selective estrogen receptor modulators (SERM) to ERα [11,12]. It was shown that GSTP protein expression occurs in the presence of ERα in drug-resistant cancer cell lines, implying that GSTP may play a role in the activation of ERα signaling leading to an abnormal response to chemotherapeutic agent such as tamoxifen.

In the present study, we demonstrate that the ectopic expression of functionally complete GSTP enhances the classical ERα signaling activities in ERα-positive but GSTP protein-negative human mammary epithelial cancer MCF7 cells. ERE-mediated ERα transcriptional activities were increased in the presence of GSTP and GSTP enzyme inhibitor decreased the ERE-mediated ERα transcriptional activities. One of molecules involved in GSTP-induced modulation of ERα signaling has been identified as receptor interacting protein 140 (RIP140). Our study proposes a novel non-enzymatic role of GSTP in regulating ERα classical signaling events, which supports the idea that GSTP has additional nonenzymatic functions other than regulation of kinase signaling pathways.

2. Materials and methods

2.1. Chemical and reagents

All chemicals and reagents were purchased from Sigma (St. Louis, MO) unless stated otherwise. Ezatiostat hydrochloride was purchased from APExBio (Boston, MA). The compounds were dissolved in DMSO and stored at −20 °C.

2.2. Cell culture

All cell culture reagents were purchased from Invitrogen (Grand Island, NY) unless stated otherwise. The MCF7 human breast cancer cell line (ATCC® HTB-22™) and HeLa human cervical cancer cell line (ATCC® CCL-2™) were obtained from American Type Culture Collection (Manassas, VA). Cells were routinely maintained in DMEM (HyClone, GE Healthcare Life Sciences, Logan, UT) containing 10% fetal bovine serum (FBS), 1% nonessential amino acids at 37 °C in 5% CO_2. Estrogen-free media were prepared by supplementing 3 × dextran-coated charcoal-treated FBS to phenol-red free DMEM (HyClone) while other components remained the same.

2.3. Plasmids and transfection

The sequences for the primers and siRNA oligonucleotides are listed in Table S1. The expression vector coding for GSTP (GenBank Accession No. NM_000852.3) was generated by inserting the 0.6 kbp long full-length GSTP into the EcoRI/BamHI sites of pIRES-neo vector (Clontech, Mountain View, CA) to produce the pIRES-neo/GSTP plasmid. The ERE-luciferase plasmid contains three copies of the *Xenopus laevis vitellogenin* A2 ERE upstream of the fire fly luciferase [13] and is a gift from Dr. V.C. Jordan (Lombardi Comprehensive Cancer Center, Georgetown University Medical Center, Washington, DC). The pSG5 plasmid containing the wild-type human ERα was originally engineered by Professor P. Chambon (Institut National de la Sante et de la Recherche Medicale, Strasbourg, France). Knockdown of the target gene was performed through the transfection of siRNA duplexes to cells. siRNA oligonucleotides with TT overhang to the end of 3′ position were purchased from Bioneer (Daejeon, South Korea). Scrambled inhibitory RNA (siControl) was derived from a message transcribed from the chloroplast genome of *Euglena gracilis* (GenBank Accession No. X70810; position 24750–24768). HeLa cells were incubated in estrogen-free media for 24 h before seeding in the 24-well plates. siRNA duplexes (30 nM) were transfected using Lipofectamine RNAiMAX reagent (Invitrogen) according to manufacturer's instruction. Six hours after transfection, the cells were put into fresh estrogen-free media and incubated for another 18 hr. ERE-luciferase plasmids (0.25 μg/well) were transfected using Lipofectamine 2000. MCF-7 or HeLa cells were cultured in estrogen-free DMEM media for 4 days before transfection and plated (1.5×10^5 cells/well) in triplicate in a 24-well plate. Cells were transiently transfected with pIRESneo/GSTP (0.5 μg/well) and ERE-luciferase plasmid (0.25 μg/well) and with an internal control plasmid pRL-Tk (0.1 μg/well) using Lipofectamine 2000 Reagent (Invitrogen).

2.4. Luciferase reporter assay

The cells were harvested with Passive Lysis buffer (Promega, Madison, WI). Luciferase activity present in the cell lysates was measured using the Dual Luciferase Assay kit (Promega) with a VICTOR3™ (PerkinElmer, Waltham, MA) in a 96 well plate format. Data are reported as relative luciferase activity (firefly luciferase reading divided by the Renilla luciferase reading).

2.5. Total RNA isolation and PCR analysis

The MCF7 cells grown in estrogen-free media were treated with appropriate test compounds or vehicle control. Total RNA was extracted using RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Total RNA (5 μg) was reverse transcribed with iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Real-time PCR was performed at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s for 30–35 cycles (Palm-Cycler™, Corbett Life Science, Sydney, Australia) in a reaction mixture with a total volume of 10 μl using the real-time PCR SYBR green kit (QIAGEN). Rotor-Gene 6.1 software was used for the estimation of the CT parameter. The $2^{-\Delta\Delta}$CT method was applied for the quantification of the gene of interest. Data normalization was performed by dividing the expression level of a *RIP140* gene by that of *18S rRNA*. Finally, results were expressed as fold-induction where the gene expression level in vehicle control-treated cells was set as 1.

2.6. Western blot

Cells were trypsinized, pelleted, washed in PBS, resuspended in lysis buffer (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 10 mM β-glycerophosphate, 10% glycerol, and 0.5% NP-40, pH 8.0) containing protease inhibitors (Complete protease inhibitor cocktail tablets, Roche, Indianapolis, IN), mixed, and centrifuged at 12,000×*g* for 10 min. Protein concentrations were measured by Bradford assay (Bio-Rad). Equal amounts of protein were electrophoresed on a 10% polyacrylamide gel and transferred to a PVDF membrane using Trans-Blot Turbo transfer system (Bio-Rad). The membrane was blocked in TBST containing 10% nonfat dry milk at 4 °C overnight and then incubated for 2 h with anti-human GSTP (3F2) mouse antibody (dilution 1:1000, Cell Signaling Technology, Danvers, MA), rabbit anti-RIP140 antibody (dilution 1:200, Santa Cruz Biotechnology, Dallas, TX) or rabbit anti-ERα

(HC-20) antibody (dilution 1:1000, Santa Cruz Biotechnology) in TBST containing 2.5% nonfat dry milk. The membranes were then incubated in TBST containing 2.5% nonfat milk and the second anti-mouse IgG (dilution 1:1000) or anti-rabbit IgG (dilution 1:3000) antibodies for 2 h. The specific proteins were detected using enhanced chemiluminescence Western blotting analysis system (Amersham ECL, GE Healthcare Life Sciences, Pittsburgh, PA) and photographed using a LAS-3000 Image analyzer (Fujifilm Life Science, Tokyo, Japan).

2.7. Data analysis and statistics

Experimental conditions were performed in duplicate or triplicate and results are representative of multiple independent experiments. All results were graphed ± standard error. Data were analyzed with an *F* test of variance to determine if the variances among groups were significantly different or not. If samples were homogenous in variance, analysis of variance (ANOVA) followed by a Student's *t*-test was applied to find statistically significant differences among treatments. Statistical significance was set at a 95% confidence interval with a $p < 0.05$ and represented by * in the figure.

3. Results and discussion

3.1. Expression of GSTP protein increases the ERα and ERE-mediated transcriptional activities

In order to investigate the effects of GSTP protein on ERα signaling events, the GSTP protein was ectopically expressed using the pIRES-neo expression plasmid (empty vector; EV) carrying human full-length wild-type GSTP gene. GSTP protein expression was positively correlated with the amount of DNA transfected as shown in Fig. 1A, whereas the major change in ERα protein level was not observed. The status of endogenous expression in both GSTP and ERα in MCF7 cells can be found in Supplementary Fig. 1A and B in which GSTP is not expressed in MCF7 cells at the protein level as reported elsewhere [14]. The ERα and ERE-mediated transcriptional activities by E_2 (1 nM) in MCF7 cells treatment were clearly increased by the transfection of GSTP expression plasmid DNA in a DNA amount-dependent manner (Fig. 1B). The transcriptional activity was approximately two-fold higher when 0.75 μg of GSTP expression DNA was transfected in cells compared to the empty vector plasmid. The ERE-luciferase activity observed under these was blocked by the ER antagonist 4-hydroxytamoxifen (4-OHT), implying that the ERE-luciferase activities observed in this experiment was clearly mediated by ER (Fig. 1C). In addition, inhibition of GSTP gene expression by siRNA treatment blocked the ERα and ERE-mediated transcriptional activities in HeLa cells, which do not endogenously expresses ERα but dose express the GSTP protein (Fig. 1D and Supplementary Fig. 1A and B). Taken together, the presence of the GSTP protein in MCF7 or HeLa cells is attributed to activation of ERα and ERE-mediated transcription and these results support our hypothesis for h the novel nonenzymatic role of GSTP as an ERα-mediated transcription modulator.

3.2. The classical ERα signaling pathway is activated in the presence of GSTP protein in E_2 concentration-dependent manner

The effect of GSTP protein expression on the ERα classical signaling pathway was further studied to determine its effect on the efficacy and/or potency of E_2 concentration-transcription response. The classical ERα signaling pathways leading to target gene expression such as the luciferase in our study design typically exhibit a concentration-dependent response after being treated with an ER agonist or antagonist that produces a Sigmoidal dose-response curve. The ectopic expression of GSTP also gives rise to a typical dose-response curve similar to that observed in EV-transfected cells (Fig. 2). As shown in Fig. 1B and C, the efficacy of E_2-mediated gene transcription response was 1.6-fold higher in the presence of GSTP. The potency as determined by EC_{50} values was not greatly affected by the GSTP expression as shown in Table 1. These data imply that the presence of GSTP may affect the degree of saturation

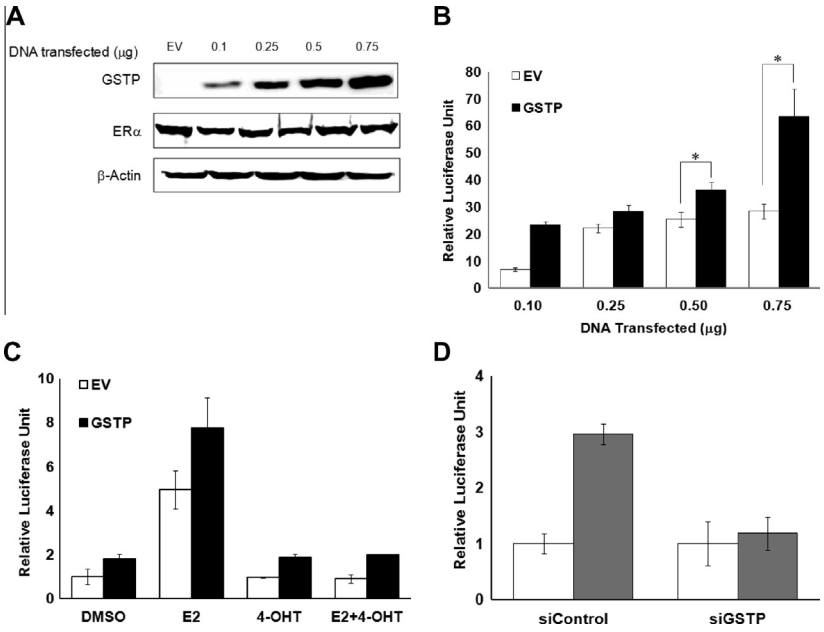

Fig. 1. Overexpression of GSTP increases the ERα and ERE-mediated transcriptional activities. Either empty vector (pIESneo plasmid; EV) or pIRESneo/GSTP (GSTP) plasmid DNA was transfected in MCF7 cells with various amounts of DNA (0.1, 0.25, 0.5 and 0.75 μg). (A) Western blot analysis of GSTP and ERα in a DNA amount-dependent manner. (B) ERE-luciferase activities by E_2 treatment in GSTP-transfected MCF7 cells. (C) Inhibition of ERα-mediated ERE luciferase activity by 4-OHT in GSTP-transfected MCF7 cells. (D) Effect of GSTP knockdown on the ERα and ERE-transcriptional activities in HeLa cells.

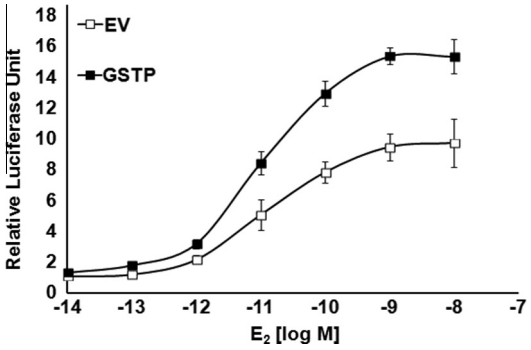

Fig. 2. Effect of GSTP expression on the E_2 concentration-dependent ERα classical signaling pathway. EV; empty vector (pIRESneo plasmid).

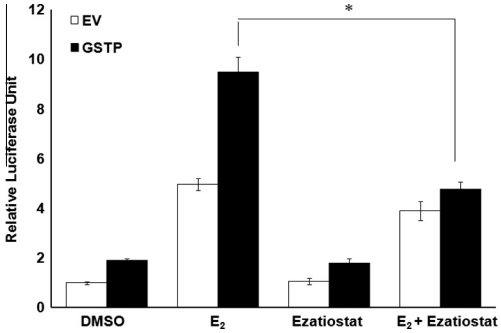

Fig. 3. Inhibition of GSTP-induced ERα transactivation by ezatiostat. At post 24-h transfection, cells were pretreated with ezatiostat (5 μM) 6 h prior to E_2 treatment.

Table 1
The effects of GSTP type on the ERα transactivation.

GSTP type overexpressed in MCF7 cells	% activity at E_2 (1 nM)	EC_{50}[a] (M)
Blank (EV)	60	1.25×10^{-11}
WT	100	1.04×10^{-11}
C47A	30	8.33×10^{-12}

[a] Half-maximal concentration of E_2 in ERE-luciferase activities.

of the available binding sites of ERα and/or activities of coregulatory proteins or other transcription factors.

3.3. Functionally active GSTP is required for its effect on the ERα and ERE-mediated transactivation

Cysteine residues present in GSTP are known to affect the structural and functional integrity of the enzyme since its catalytic activity was only partially reduced upon cysteine modification [15,16]. In particular, cysteine 47 is shown to be the thiol group most sensitive to chemical modification and its replacement with alanine lead to a ~34% reduction in enzymatic activity. In order to investigate the structural requirement of GSTP-induced ERE-reporter transcriptional activity, the plasmid coding the mutant GSTP/C47A was co-transfected with ERE-luciferase plasmid to MCF7 cells and the luciferase activities were monitored. The E_2-mediated luciferase activity was dramatically decreased by 70% when the GSTP/C47A mutant was expressed whereas the there was no significant change in EC_{50} compared to GSTP/WT (Table 1).

In addition, treating the cells with Ezatiostat hydrochloride (Telintra®; TLK199), a GSTP1-1-specific inhibitor [17], also decreased the GSTP-induced ERE-luciferase activities by 50% compared to 21% in EV-transfected cells (Fig. 3). These data suggest that the functional integrity of GSTP is a prerequisite to the induction of GSTP-induced ERα transcriptional activity. In addition, our results propose an additional value of GSTP inhibitors as an intervention measure to modify several disease conditions associated with ERα classical signaling activities.

3.4. GSTP affects the expression of receptor interacting protein 140 at both mRNA and protein levels

Transcriptional efficiency of the nuclear receptor-associated gene expression is highly and tightly regulated by the function of coregulatory proteins in forming the initiation complex [18]. To further investigate the molecular mechanisms of action for GSTP-induced ERα classical signaling, we performed experiments to determine if GSTP affects the expression of the coregulatory proteins involved in ERα-mediated transcription processes in the presence of E_2 in MCF7 cells. It was observed that RIP140 was down-regulated upon E_2 treatment of cells transiently transfected with GSTP expression plasmid compared to those with EV at both the mRNA and protein levels as shown in Fig. 4. RIP140, also known as nuclear receptor interacting protein 1 (NRIP1), is a negative transcriptional regulator of nuclear receptors and a cofactor recruited by ERα activating function-2 (AF-2) domain in the presence of ligand [19]. The strong inhibitory action of RIP140 on ERα-target gene transcription was attributed to its competition with coactivator binding to ERα and its recruitment of various

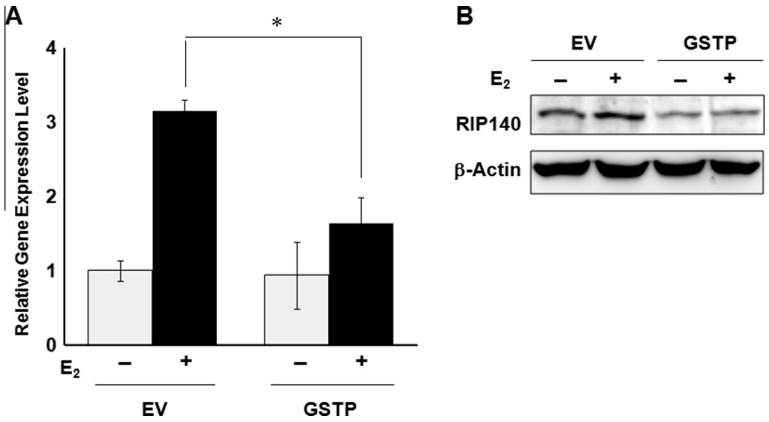

Fig. 4. RIP140 expression at both the mRNA and protein levels by ectopic expression of GSTP and E_2 treatment. The mRNA level of RIP140 was measured using real-time PCR analysis and the protein level was studied using Western blot analysis.

repressive effectors [20,21]. It is not known whether GSTP directly modulates the expression of the RIP140 gene or protein since GSTP is not a known transcription factor or modulator. The binding partners or transcription factors involved in RIP140 gene expression have already been identified and do not include GSTP. The mechanism by which GSTP down-regulates RIP140 may involve the anti-oxidative effect by GSTP, since it is known that RIP140 overexpression causes mitochondrial dysfunction and produces reactive oxygen species and protection against such oxidative stress via GSTP modulates RIP40 expression [22].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.09.017.

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