Activation of Signal Transduction Kinases by Tamoxifen

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Purpose. To study the signal transduction mechanisms of tamoxifen via the activation of MAPKs, JNK and ERK in order to understand its regulation of gene expression.

Methods. The effects of tamoxifen (TAM) on the activation of serine/threonine mitogen-activated protein kinase (MAPK, p42/ERK2) and the stress-activated protein kinases (p46 SAPK or c-Jun *N*-terminal kinase, JNK1) were evaluated using a human cervical epitheloid carcinoma HeLa cell line.

Results. TAM activated both JNK1 and ERK2 activities in a time- and dose-dependent manner in HeLa cells. The activation of JNK1 was enhanced when the cells were pretreated with prooxidant H_2O_2.

Conclusions. These studies show that TAM activates the signal transduction kinases, JNK1 and ERK2, which may play important roles in the regulation of gene expression by TAM.

KEY WORDS: tamoxifen; MAP kinases; MAPK; JNK; ERK; AP-1; ARE; NF-κB; signal transduction.

INTRODUCTION

The anti-estrogen drug tamoxifen (TAM) [trans-1-(4β-dimethylaminoethoxyphenyl) 1,2-dephenybut-1-ene], is presently the most widely used therapeutic agent for the treatment and chemoprevention of breast cancer (1–3). TAM has recently been shown to induce a number of drug metabolizing enzymes including phase I cytochrome P450s and phase II enzymes such as glutathione *S*-transferases (GST), epoxide hydrolase (EPH), and quinone reductase (QR), as well as certain isoforms of sulfotransferase (ST) and UDP-glucuronosyltransferase (UGT) (4–5). Furthermore, TAM has been shown to transcriptionally activate a 1.6 kilobase (kb) 5′-flanking region of the rat GSTya subunit gene expressed in a human hepatoma Hep G2 cell line (6). The regulation of drug metabolizing enzymes plays an important role in the biotransformation of many pharmaceutical agents as well as in toxicology of many environmental pollutants and carcinogens. The common use of TAM in the clinical settings also opens the possibility of certain drug-drug interactions which might either enhance or diminish the biological activity of these drugs. Additionally, the benefits of antiestrogens therapies in osteoporosis, breast cancer, and heart disease are well documented, however, the exact molecular mechanisms remains elusive (7). TAM, and other antiestrogens such as ICI 164,384, and raloxifen, have been developed as antiestrogens, which antagonize estrogen actions in reproductive tissues. These compounds inhibit 17β-estradiol (E_2)-induced activation of estrogen-response element (ERE)-containing genes to various extents (8).

To date, the mechanism underlying the regulation of gene expression of many phase II genes by xenobiotics or by TAM remains unknown. However, since many of these drug metabolizing enzymes lack ERE in their 5′-flanking regions, additional signaling and/or regulatory mechanisms shall exist. Indeed, recently TAM has been found to activate AP-1 pathway (9), modulates protein kinase C via oxidative stress in estrogen receptor negative breast cancer cells (10), and another antiestrogen, raloxifen has been shown to activate a newly defined raloxifen response element (RRE) in the promoter region of transforming growth factor-β3 (TGFβ3) (11).

In the present study, the effects of TAM were investigated on the signal transduction mechanism by evaluating the activation of an important family of signaling molecules, the mitogen-activated protein kinases (MAPKs). Responses to numerous types of extracellular signals are mediated by MAPKs which are members of a serine/threonine kinase family (12). A well defined MAPK subfamily are extracellular regulated kinases (ERK1 and ERK2), which are responsible for the phosphorylation and activation of various transcription factors, including c-Myc and TCF/Elk1 (13). Another emerging group of MAPKs are c-Jun *N*-terminal kinases (JNK1 and JNK2), also called stress-activated protein kinases (SAPKs), since they can be stimulated by a variety of stresses (14). JNK activity can be induced by diverse stimuli such as growth factors, cytokines, T-cell activators, certain protein synthesis inhibitors, UV irradiation, γ-irradiation, heat shock, and osmotic shock (14–20). Once activated, JNK can phosphorylate various transcription factors such as AP-1, ATF2 and TCF/Elk-1 leading to immediate-early gene induction (21–23). Activation of JNKs by such a wide range of factors indicates that this cascade may serve as a common signaling pathway and can integrate with other numerous signals. In this report our data showed that TAM activates the MAPKs, JNK1 and ERK2 in a concentration- and time-dependent fashion in HeLa cells.

MATERIALS AND METHODS

Cell Culture

Human cervical epitheloid carcinoma HeLa cells obtained from ATCC (Rockville, MD) were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum and antibiotics as described in our previous publication (24). Cells were serum starved for 16 h and then exposed to various stimulations as indicated.

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ABBREVIATIONS: TAM (tamoxifen), JNK (c-Jun *N*-terminal kinase), MAP kinase (mitogen-activated protein kinase), ERK-1 and 2 (extracellular signal-regulated kinase 1 and 2), ARE (antioxidant responsive element), EpRE (electrophile responsive element), AP-1 (activator protein-1) and NF-κB (nuclear factor kappa B), GSH (glutathione), GST (glutathione *S*-transferases), QR (quinone reductase), ST (sulfotransferase), UGT (UDP-glucuronosyltransferase), MBP (myelin basic protein), PMSF (phenylmethylsulfonyl fluoride), TPA (12-*O*-tetradecanoylphorbol-13-acetate), tBHQ (tert-butylhydroquinone), ROI (reactive oxygen intermediate), IP (immunoprecipitation).

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Materials and Reagents

Anti-ERK2 antibody and protein A Sepharose 4B conjugate were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-JNK1 (JNK 1 [a.a. 368–384]) was generated by immunized rabbits with 17-mers JNK peptide conjugated to KLH as described previously (20). [γ-^{32}P] ATP (5,000 Ci/mmol) was purchased from NEN-Dupont (Boston, MA). MBP, anisomycin, tamoxifen, diamide, PMSF, TPA, and tBHQ, were purchased from Sigma Chemical Co. (St. Louis, MO).

Preparation of Cell Extracts

After stimulations as indicated, cells were scraped off the plates and lysed in the lysis buffer containing 10 mM Tris-base, pH 7.1, 50 mM NaCl, 50 mM sodium fluoride, 30 mM sodium pyrophosphate, 100 μM sodium orthovanadate, 2 mM iodoacetic acid, 5 uM $ZnCl_2$, 0.1% BSA (used only in immunoprecipitation), 1 mM PMSF (added fresh), and 0.5% Triton-X-100 as described previously (25). The lysate was homogenized by passing through a 25 G needle three times and the homogenate was centrifuged at 14,000 rpm for 10 min at 4°C. The supernatant cell lysate was carefully transferred to a clean tube and stored at −20°C until analysis within one-week period. Protein concentrations were estimated by the BCA method (Pierce Chemical Co., Rockford, IL).

IP and *in Vitro* Immunocomplex Kinase Assay of ERK2

IP was performed by the addition of anti-ERK2 antibody (10 μg for each sample) to protein A-Sepharose (30 μl for each sample) and rotated at 4°C for 16 hr. The complex was then washed twice with ice-cold phosphate buffered saline (PBS) and the supernatant cell lysate was added and immunoprecipitated with rotation at 4°C for 1 hr. The immunocomplexes were washed twice with lysate buffer, and twice with kinase buffer containing 20 mM Hepes, pH 7.9, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 50 mM β-glycerolphosphate, and 10 mM *p*-nitrophenyl phosphate as described previously (25). The immunocomplex kinase assay was initiated by the addition of 30 μl cocktail containing 2 μCi [γ-^{32}P]ATP, 10 μM ATP, and 10 μg MBP in a solution containing 60% kinase buffer/40% PBS and incubated for 15 min at 30°C. The kinase reaction was terminated by the addition of 90°C-sample loading buffer, and incubated at 95°C for 5 min. After high speed (14,000 rpm for 5 min at 4°C) centrifugation, the supernatants were resolved in 14% SDS-PAGE. After washing in 30% methanol/10% acetic acid solution overnight, the ^{32}P-phosphorylation of the MBP protein was visualized by autoradiography and quantitated with a phosphoimaging analyzer (AMBIS, San Diego, CA).

IP and *in Vitro* Immunocomplex Kinase Assay of JNK1

The procedures for JNK1 assay were similar to that described above for ERK2 assay with slight modifications as follows. After IP of cell lysates with anti-JNK1 antibody, the in vitro immunocomplex kinase reaction was carried out at 30°C for 30 min, with each sample containing 10 μg of *GST-c-Jun* (1–79) protein as the substrate for JNK1. The phosphorylated *GST-c-Jun* was resolved in 10% SDS-PAGE gel.

RESULTS AND DISCUSSION

TAM has been shown to induce various drug metabolizing enzymes including phase I cytochrome P450s and phase II GST, QR, ST and UGT in rat livers (4,5). To date, the mechanisms of induction are not well understood. Utilizing three human cell lines, we investigated the activation of an important family of signaling molecules, the MAPKs (ERK2 and JNK1) by TAM. [All experiments have been repeated at least three times, and the representative data are presented below]. Fig. 1 shows the dose response of TAM treatments (30 min for ERK2 and 60 min for JNK1) on the activation of ERK2 (A) and JNK1 (B).

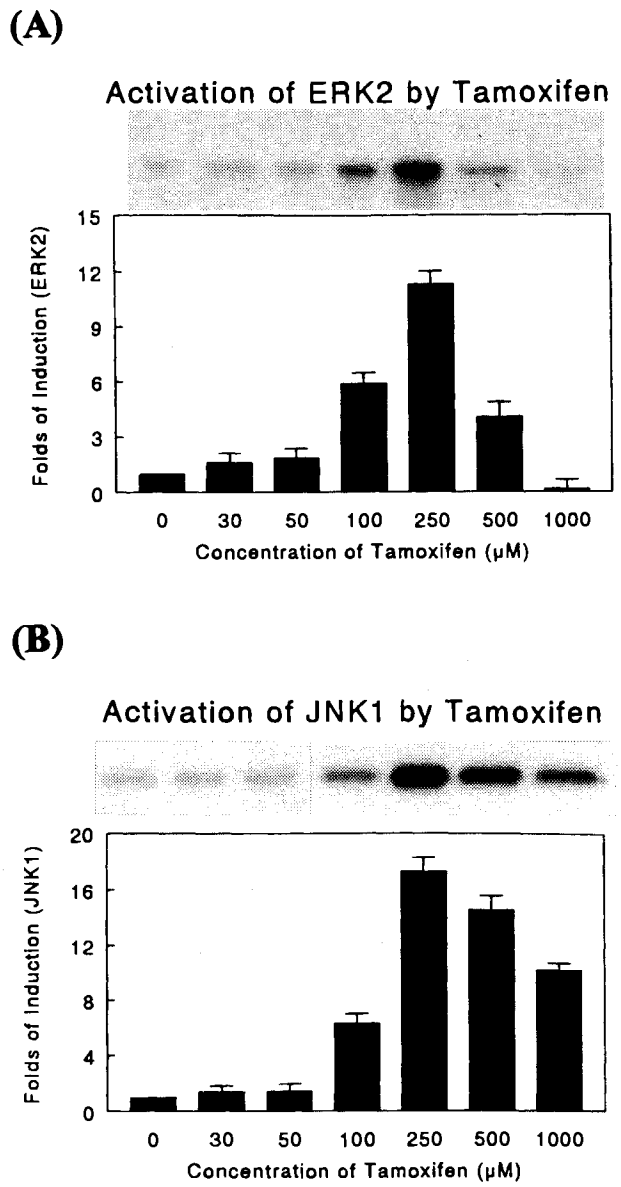

Fig. 1. Effects of TAM on ERK2 and JNK1 activities. Dose response of (A) ERK2 (30 min), and (B) JNK1 (60 min) activities in HeLa cells. Both activities present in cell lysates were determined by immunocomplex kinase assay using 10 μg of myelin basic protein (MBP) or 10 μg *GST-c-Jun* (1–79), respectively. The kinase reaction was carried out at 30°C for 15 min (ERK2) or 30 min (JNK1) in the presence of 2 μCi [γ-^{32}P]ATP and the products were resolved in 14% (ERK2) or 10% (JNK1) SDS-polyacrylamide gel electrophoresis.

The activation of ERK2 and JNK1 by TAM both peaked at 250 μM. Further increase in TAM concentrations led to a decrease of both kinase activities, although ERK2 decreased more rapidly. At 1 mM concentration of TAM, ERK2 activity was below that of the control level, whereas JNK1 activity was still substantially elevated compared to that of the control. The decrease in both kinase activities could be due to toxic effects of TAM at these high concentrations and therefore, all subsequent experiments were conducted with TAM at concentrations below 250 μM.

Figure 2 shows the kinetics of ERK2 (A) and JNK1 (B) activation with 100 μM of TAM in HeLa cells. ERK2 activity peaked at 30 min, decreased slightly at 60 min, and it was below the level of control cells after 4 hr of TAM treatment (data not shown). In contrast, JNK1 activity peaked at 60 min, decreased slightly at 90 min and it was sustained up to 4 hr (data not shown).

Since JNK1 activity can be activated by diverse stress stimuli including oxidative stress with H_2O_2 (26), we asked the question whether pretreatment with different oxidative stress chemicals (H_2O_2 and diamide) would have any effects on TAM's activation of ERK2 and JNK1. Fig. 3A shows that both H_2O_2 and diamide activated ERK2 activity alone and pretreatment of cells with either H_2O_2 or diamide had no substantial effects

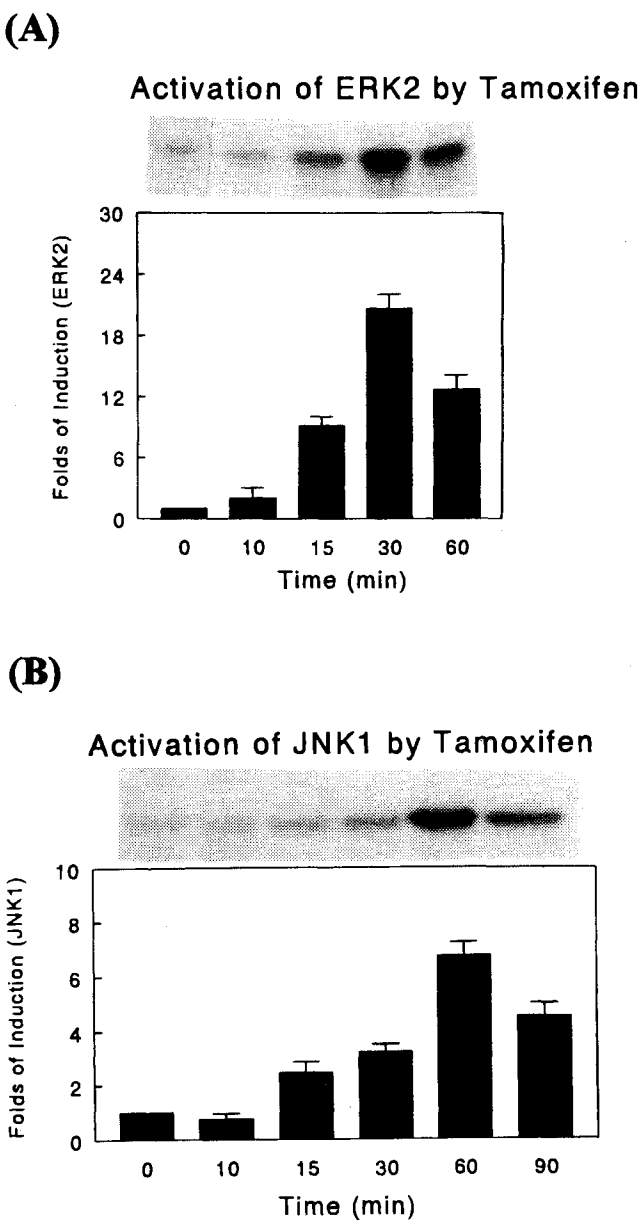

Fig. 2. Effects of TAM on ERK2 and JNK1 activities. Time course of (A) ERK2 and, (B) JNK1 activities in HeLa cells treated with TAM (100 μM). Both activities present in cell lysates were determined by immunocomplex kinase assay using 10 μg of myelin basic protein (MBP) or 10 μg *GST-c-Jun* (1–79), respectively.

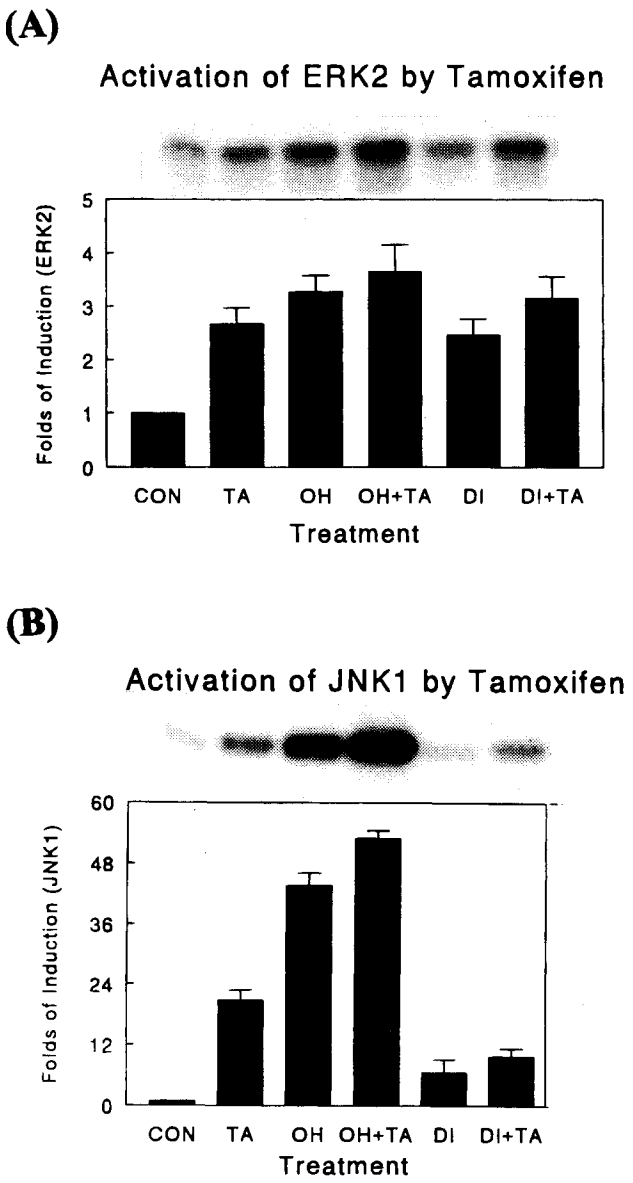

Fig. 3. Effect of TAM (TA), H_2O_2 (HO), and Diamide (DI) on the activation of ERK2 and JNK1 activities. (A) Lanes: 1, control; 2, TAM (100 μM, 30 min); 3, H_2O_2 (200 μM, 60 min); 4, H_2O_2 (200 μM, 30 min) followed by TAM (30 μM 30 min) in PBS buffer; 5, diamide (1 mM, 3 min); 6, diamide (1 mM, 3 min), followed by TAM (30 μM, 30 min) in PBS buffer. Both activities present in cell lysates were determined by immunocomplex kinase assay using 10 μg of myelin basic protein (MBP) or 10 μg *GST-c-Jun* (1–79), respectively.

on TAM-mediated ERK2 activity. Figure 3B shows that H_2O_2 alone activated JNK1 activity, and H_2O_2 potentiated JNK1 activation by TAM (lane 4). Diamide alone slightly activated JNK1 activity but unlike H_2O_2, diamide inhibited TAM's activation of JNK1. Diamide is a prooxidant that reacts with thiols of GSH (27), or converts reduced GSH to oxidized glutathione (GSSG) (28). The depletion of GSH will make conditions in the cell more oxidizing, and diamide would therefore be expected to mimic the effect of H_2O_2. However, different effects were observed between H_2O_2 and diamide as demonstrated by our study as well as by others (29). Further study would be needed to reveal the mechanism. Nevertheless, oxidative stress with the perturbation of GSH and GSSG levels in the cells, had an impact on the signaling by TAM especially via the JNK pathway, and similar findings on the role of oxidative stress in modulation of protein kinase C had been reported recently (10). Using reverse-transcription-polymerase chain reaction (RT-PCR), TAM could indeed induce c-jun mRNA expression in HeLa cells (data not shown). Our findings agreed with published findings that TAM induced proto-oncogene c-fos, jun-B, jun-D expression in uterine endometrial epithelium (9).

In summary, this is the first report to demonstrate that TAM activated an important family of signaling molecules, the MAPKs, ERK2 and JNK1. The exact mechanism of activation by TAM remained to be elucidated, but it may involve GSH and/or GSSG. Since these kinases play critical role in phosphorylation and activation of various transcription factors leading to immediate-early gene induction, it is tempting to speculate that TAM may regulate various gene expression including various drug metabolizing enzyme genes via these two kinase pathways. Questions remain as to what transcription factors and/or DNA binding elements are activated by TAM through these kinase cascades. Furthermore, at this point we can not rule out the involvement of the estrogen receptor in the activation by TAM. However, preliminary studies utilizing breast carcinoma cell line, BT-20 which lacks estrogen-receptor, showed that TAM activated both JNK1 and ERK2 (unpublished observations), suggesting that activation of these kinases may not require estrogen receptor. In conclusion, our studies demonstrate that TAM activates the signal transduction kinases, JNK1 and ERK2 which may play important roles in the regulation of gene expression by TAM.

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