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A Possible Involvement of Tyrosine Kinase in TRH-induced Prolactin Secretion in GH3 Cells

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SUMMARY: Thyrotropin-releasing hormone (TRH) is a well-known regulatory factor of prolactin (PRL) secretion and synthesis in lactotrophs. Recently we have found that TRH stimulates early tyrosine phosphorylation of MAP kinase in GH3 cells. Then we investigated whether tyrosine phosphorylation in TRH action is involved in TRH-stimulated PRL secretion by GH3 cells, using a 4-hydroxycinnamamide derivative (ST638), a tyrosine kinase inhibitor. TRH-stimulated tyrosine phosphorylation of MAP kinase and PRL secretion were remarkably inhibited by ST638 treatment. These results suggest that tyrosine phosphorylation of MAP kinase is strongly associated with TRH-stimulated PRL secretion.

TRH is a well-known regulatory factor of prolactin (PRL) secretion and synthesis in lactotrophs. The association of TRH with a specific membrane receptor (1) triggers a sequence of events that include the interaction of the hormone-receptor complex with a G protein (2). This is followed by the activation of phospholipase C with subsequent stimulation of phosphoinositide hydrolysis (3) and generation of second messengers including inositol phosphates (IPs) (4), protein kinase C (PKC) (5), intracellular Ca^{2+}, arachidonate (6) and so on.

Also phosphorylation of intracellular proteins has been considered to play an important role in regulating events throughout intracellular signaling cascades, and some studies on phosphorylation of cellular proteins during TRH stimulation have been reported. For instance, TRH was revealed to increase the phosphorylation of a specific group of proteins in the normal anterior pituitary, GH3 cells and GH4C1 cells (7,8). In spite of these attempts, little was known regarding the relationship between TRH-stimulated PRL secretion and phosphorylation of intracellular proteins. Recently we have found that TRH stimulates early tyrosine phosphorylation of MAP kinase in GH3

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cells (9). Microtubule-associated proteins (10) have been proposed as substrates for MAP kinase, and they may be involved in hormone secretion (11). In the present study, using a 4-hydroxycinnamamide derivative (ST638) (12), a tyrosine kinase inhibitor, we investigated whether tyrosine phosphorylation of MAP kinase is involved in TRH-stimulated PRL secretion in GH3 cells.

Materials and Methods

Materials. ST638 was a generous gift from Kanegafuchi Chemical Industry Co.(Hyogo, Japan). TRH was purchased from Peninsula Laboratories Inc. [^{125}I]-Labeled anti-mouse immunoglobulin (Ig) was obtained from Amersham (Arlington Heights, IL). Anti-phosphotyrosine antiserum was obtained from Upstate Biotechnology (Lake Placid, NY). Polyclonal anti-MAP kinase antiserum was from Santa Cruz Biotechnology.

Immunoblotting. For the immunoprecipitation of MAP kinase, GH3 cells were seeded into 60mm dishes and grown with Ham F10 containing 15% horse serum and 2.5% fetal bovine serum in a humidified 37C atmosphere of 5% CO_2 and 95% air. Before TRH-stimulation, the medium was replaced by serum-free medium with or without ST638 (50μM) and incubated for 1hr. 1μM TRH was directly added to the medium and incubated for 2.5 min at 37C. After treatment, the cells were washed once with phosphate-buffered saline (PBS), followed by the addition of 500μl of HNTG buffer (50mM HEPES, pH7.5, 150mM NaCl, 10% glycerol, 1% TritonX-100, 1.5mM $MgCl_2$, 1mM EDTA, 10mM sodium pyrophosphate, 100μM sodium orthovanadate, 100mM NaF, 10μg/ml aprotinin, 10μg/ml leupeptin, and 1mM phenylmethylsulphonyl fluoride) (9). Lysates were centrifuged at 10,000xg for 10min. Supernatants were incubated for 1hr with polyclonal anti-MAP kinase antiserum. Following the incubation, protein A-Sepharose beads were added for 30min with mixing and immune complexes bound to the beads were washed three times with HNTG buffer. Immune complexes were solubilized in 25μl Laemmli SDS sample buffer (13). Samples were heated at 95C for 5min, and 30μg protein aliquots were loaded onto 8% SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose paper and immunoblotted with anti-phosphotyrosine antiserum as described (14).

PRL secretion. GH3 cells were seeded into Falcon 24-well plates, grown under the same conditions as described above. When the cell growth reached confluence, cells were preincubated with various concentrations of ST638 for 1 hour and stimulated with TRH (1μM) without changing the concentration of ST638. After 30min, the medium was collected and the concentration of PRL was determined by double-antibody RIA, using the materials and protocols supplied by the NIDDK, NIH (Bethesda, MD).The intra- and interassay variations for PRL were less than 8% and 10%, respectively.

Results

Effect of tyrosine kinase inhibitors on basal PRL secretion. To examine the effect of ST638 on basal secretion by GH3 cells, GH3 cells were incubated with or without 50μM ST638. One hour treatment of 50μM ST638 didn't affect basal secretion by GH3 cells (data not shown). At this concentration, ST638 had no effect on cell viability, as determined by trypan blue exclusion. Furthermore, one hour after washing out this agent, both cell lines showed good responses of PRL secretion to TRH, indicating that ST638 is not acutely toxic to GH3 cells.

Tyrosine kinase inhibitor inhibits TRH-stimulated tyrosine phosphorylation of MAP kinase. To evaluate the effect of ST638 on TRH-induced tyrosine phosphorylation, GH3 cells were preincubated with or without 50μM of ST638 for 1hr. After treatment with

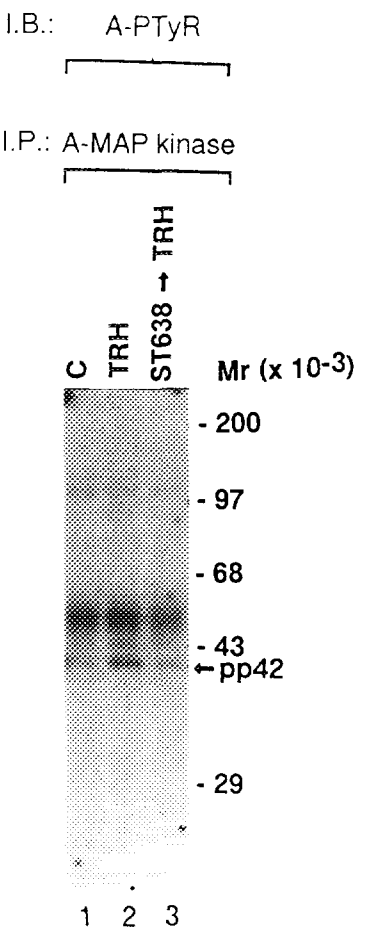
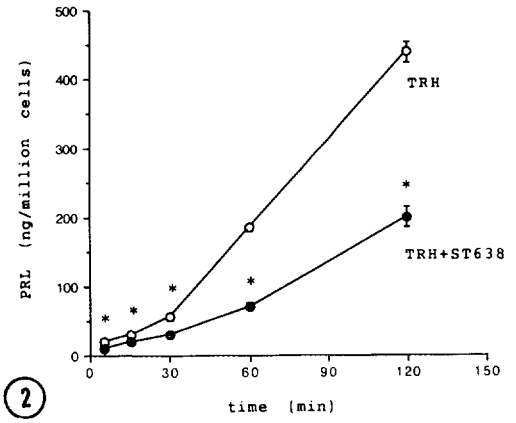


Fig. 1. TRH-induced tyrosine phosphorylation of MAP kinase. GH3 cells were grown in 60mm dishes. After 60min treatment with or without 50μM ST638, TRH (final concentration: 1μM) was added directly to the medium and incubated for 2.5min. Lysate was immunoprecipitated with anti-MAP kinase antiserum and the immunocomplexes were analyzed by SDS-PAGE followed by immunoblotting with anti-phosphotyrosine antiserum, as described in "Materials and Methods". TRH stimulates tyrosine phosphorylation of MAP kinase in GH3 cells (lane 2). ST638 remarkably attenuated the phosphorylation in GH3 cells (lane 3).

1μM TRH for 2.5min, lysates were prepared and immunoprecipitated with anti-MAP kinase antiserum, followed by SDS-PAGE and autoradiography. In GH3 cells, TRH stimulated early tyrosine phosphorylation of MAP kinase (Fig.1 lane2). The TRH-induced tyrosine phosphorylation of MAP kinase by GH3 cells was remarkably attenuated by pretreatment of the cells with ST638 (Fig.1 lane3).

Effect of tyrosine kinase inhibitors on TRH-stimulated PRL secretion. Figure 2 shows the time courses of the effects of ST638 on TRH-stimulated PRL secretion by GH3 cells. In the group subjected to ST638 treatment, the cells were preincubated for one hour with 50μM ST638 before 1μM TRH was added at time zero. Suppression of PRL secretion was observed as early as 5min after the TRH was added and continued throughout the next two hours of incubation in GH3 cells. Figure 3 shows the data on



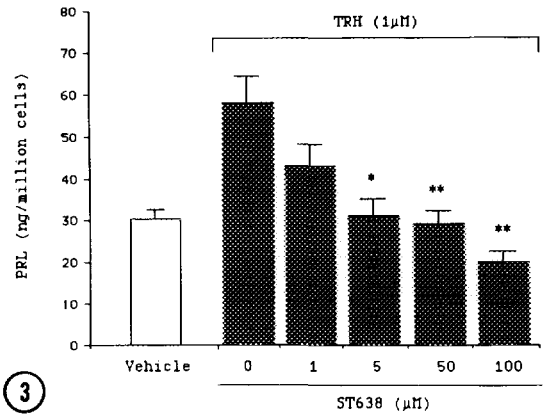


Fig. 2. Time courses of effects of ST638 (50μM) on TRH-stimulated PRL secretion by GH3 cells. In the groups subjected to ST638 treatment, the cells were preincubated for 1hour with ST638 (50μM) before TRH (1μM) was added at time zero. ST638 significantly (*P<0.01 vs. vehicle) suppressed PRL secretion at all time points. Each point represents the mean ± SE of results for six wells.

Fig. 3. Dose-dependent inhibition of TRH-stimulated PRL secretion by ST638 is illustrated in these figures. In the groups subjected to ST638 treatment, the cells were preincubated for 1hour with various concentrations the of ST638 before starting the final 30-min incubation period. Then the cells were stimulated by 1μM TRH without changing the concentration of ST638 for 30 min. Each point represents the mean ± SE of results (*P<0.05; **P<0.01).

dose-dependent inhibition of TRH-stimulated PRL secretion by ST638 in GH3 cells. In the groups subjected to ST638 treatment, the cells were preincubated for one hour with various concentrations of ST638 before starting the final 30min incubation period. The cells were then stimulated with 1μM TRH without changing the concentration of ST638.

Discussion

The phosphorylation of proteins by various protein kinases plays a significant role in the cascade of intracellular biochemical events initiated by hormones acting at the plasma membrane (15). It is now recognized that many cell surface receptors contain intrinsic tyrosine kinase activities (16), associate with tyrosine kinase (17), or activate tyrosine phosphorylation (18). Thus, tyrosine phosphorylation is believed to be the first step in many signaling pathways. Although multiple proteins were shown to be phosphorylated following addition of TRH to GH3 cells (19), they were not yet identified. The fact that epidermal growth factor (EGF) as well as TRH stimulated PRL secretion (20) inspired us to investigate the comparison of the effects of both EGF and TRH on MAP kinase activity. As a result, recently we have found that TRH stimulates early tyrosine phosphorylation and activation of MAP kinase (9) by way of another PKC-independent pathway, however, its function has not yet been examined. This is the first report to demonstrate that tyrosine kinase inhibitor

(ST638) inhibited both TRH-stimulated tyrosine phosphorylation of MAP kinase and PRL secretion in GH3 cells, suggesting that tyrosine phosphorylation of MAP kinase is involved in TRH-stimulated PRL secretion. One of MAP kinase's substrates is microtubule-associated protein (21). Moreover secretory granules are shown to be closely associated to microtubules (11,22) and agents that prevent the assembly of microtubules exert an inhibitory effect on hormone secretion (23,24). Considering these reports, microtubule-associated protein and MAP kinase are speculated to play an important role in transport and exocytosis of PRL secretary granules. We also found that TRH-stimulated tyrosine phosphorylation of MAP kinase was inhibited by dopamine (manuscript in preparation). Taken together, these facts suggest that tyrosine phosphorylation of MAP kinase is closely associated with the TRH-stimulated secretory response of PRL secretion.

ST638 is reported to inhibit the tyrosine-specific protein kinase activity of epidermal growth factor (EGF) receptors with an IC50 value of 0.37μM (12,25). Unlike other tyrosine kinase inhibitors, such as, genistain, quercetin, amiloride and ATP analogues, all of which inhibit tyrosine kinases competitively with respect to ATP, the specifity of ST638's inhibitory action is likely to be derived from the similarity of its structure to that of tyrosine residues in substrate proteins (12). Although the dose of ST638 might have been a little high in the present study, even at the concentrations up to 100μM it has been reported to show no inhibitory effect on the enzyme activities of serine- and/or threonine-specific protein kinases such as cAMP-dependent protein kinase and PKC (12). We also investigated whether tyrphostin 25 (AG 18) (26), which is another specific tyrosine kinase inhibitor, suppressed TRH-stimulated PRL secretion, and observed the same tendency (data not shown). This suggests that ST638 exerts an inhibitory effect on the tyrosine kinases, which may be related to the signal transduction pathway of the TRH-mediated secretory response.

In conclusion, a tyrosine kinase inhibitor affected the TRH-stimulated secretory response of PRL secretion and the TRH-stimulated tyrosine phosphorylation of MAP kinase. These results suggest that tyrosine phosphorylation of MAP kinase is strongly associated with TRH-stimulated PRL secretion.

Acknowledgments

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