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Thyroid-stimulating Hormone and Cyclic AMP Activate p38 Mitogen-activated Protein Kinase Cascade

INVOLVEMENT OF PROTEIN KINASE A, Rac1, AND REACTIVE OXYGEN SPECIES*

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p38 mitogen-activated protein kinases (p38-MAPKs) are activated by cytokines, cellular stresses, growth factors, and hormones. We show here that p38-MAPKs are activated upon stimulation by thyroid-stimulating hormone (TSH) or cAMP. TSH caused the phosphorylation of p38-MAPK in Chinese hamster ovary cells stably transfected with the human TSH receptor but not in wild-type Chinese hamster ovary cells. The effect of TSH was fully mimicked by the adenylyl cyclase activator, forskolin, and by a permeant analog of cAMP. The effect of forskolin was reproduced in FRTL5 rat thyroid cells. TSH also stimulated the phosphorylation of MAPK kinase 3 or 6, over the same time scale as that of p38-MAPKs. TSH and forskolin stimulated the activity of the α-isoform of p38-MAPK assayed by phosphorylation of the transcription factor ATF2. The activity of MAPK-activated protein kinase-2 was stimulated by TSH and forskolin. This stimulation was abolished by SB203580, a specific inhibitor of p38-MAPKs. The protein kinase A inhibitor H89 inhibited the stimulation of phosphorylation of p38-MAPKs by forskolin, whereas inhibitors of protein kinase C, $p70^{S6k}$, and phosphatidylinositol 3-kinase were ineffective. Expression of the dominant negative form of Rac1, but not that of Ras, blocked forskolin-induced p38-MAPK activation. Diphenylene iodonium, a potent inhibitor of NADPH oxidase(s), and ascorbic acid, an effective free radical scavenger, suppressed TSH- or forskolin-stimulated p38-MAPK phosphorylation, indicating that the generation of reactive oxygen species plays a key role in signaling from cAMP to p38-MAPKs. Inhibition of the p38-MAPK pathway with SB203580 partially but significantly, attenuates cAMP- and TSH-induced expression of the sodium iodide symporter in FRTL-5 cells. These results point to a new signaling pathway for the G_s-coupled TSH receptor, involving cAMP, protein kinase A, Rac1, and reactive oxygen species and resulting in the activation of a signaling kinase cascade that includes MAPK kinase 3 or 6, p38-MAPK, and MAPK-activated protein kinase-2.

The activation of mitogen-activated protein kinases (MAPKs)[1] is a key event in many cellular processes, including proliferation, differentiation, and apoptosis (1). There are three main classes of MAPK, extracellular signal-regulated protein kinases (ERKs) (2, 3), stress-activated protein kinases, also known as c-Jun NH_2-terminal protein kinases (JNKs) (4, 5), and p38-MAPKs (6–11). p38-MAPKs α, β, γ, and δ are activated by the dual phosphorylation of threonine and tyrosine residues within the tripeptide motif TGY (12) by MAP kinase kinases termed MKK3 and MKK6, themselves activated by phosphorylation (13). The p38-MAPKs phosphorylate other protein kinases, such as MAPKAP kinase-2/3 (14, 15) and transcription factors, such as ATF2, Elk1, Sap-1a, MEF2, and CHOP (16–20). The pyridinylimidazole compounds SB203580 and SB202190 are very specific inhibitors of p38α- and p38β-MAPKs (21, 22). The Rho family GTPases Rac1 and CDC42 and the STE20-related protein kinases PAK1, PAK3, and germinal center kinase have all been implicated in the p38-MAPK signaling pathway (23–25).

ERKs are preferentially activated in response to growth factors, cytokines, and mitogens, via the well known Ras/Raf-1/MEK pathway. JNKs and p38-MAPKs are activated in response to a variety of cell stresses, including UV irradiation, pro-inflammatory cytokines, protein synthesis inhibitors, osmotic shock, and chemical stress such as that induced by H_2O_2 and other reactive oxygen species. A variety of agonists acting on G protein-coupled receptors have also been shown to activate ERK, JNK, or p38-MAPK pathways (26). Heterogeneity exists in the mechanisms whereby G protein-coupled receptors activate MAP kinases. Much data suggest that the $\beta\gamma$ subunits resulting from the dissociation of the heterotrimeric G proteins G_s, G_i/G_o, or G_q/G11, can interact with effectors acting on the Ras-ERK pathway (27, 28) or on the JNK and p38-MAPK pathways (29, 30). On the other hand, the α subunits of G12 or G13 activate the JNK pathway (31) and the α subunits of Gq/G11 activate the p38-MAPK pathway (32).

The receptor for thyroid-stimulating hormone (TSH) is a seven-transmembrane spanning receptor that is coupled to G_s and, in the human thyroid, to G_q/G11 (33). Activation of G_s

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[1] The abbreviations used are: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated protein kinase; JNK, c-Jun NH_2-terminal protein kinase; MKK, MAPK kinase; MAPKAP kinase-2, MAPK-activated protein kinase 2; PI3K, phosphatidylinositol 3-kinase; PKA, cAMP-dependent protein kinase; TSH, thyroid-stimulating hormone; Fk, forskolin; HA, hemagglutinin; GST, glutathione *S*-transferase; DPI, diphenyleneiodonium; PAGE, polyacrylamide gel electrophoresis; ROS, reactive oxygen species; CHO, Chinese hamster ovary; NIS, sodium iodide symporter; FCS, fetal calf serum; EGF, epidermal growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; rchTSH, recombinant human TSH; hTSHR, human TSH receptor.

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leads to the stimulation of adenylyl cyclase and activation of the cAMP-dependent protein kinase (PKA). cAMP is a positive modulator of thyroid cell differentiation and proliferation. The direct activation of the cAMP pathway by forskolin, cholera toxin or cAMP analogs reproduces most of the effects of TSH (34, 35). In thyroid cells, TSH stimulates the expression of sodium iodide symporter (NIS) through the cAMP pathway (36), as well as that of other differentiation markers such as thyroperoxidase and thyroglobulin. However, the biochemical basis for the stimulatory effect of TSH and cAMP is not completely elucidated. Members of the CRE-binding (CREB) family of cAMP-responsive transcription factors are required but do not appear to be sufficient to fully mimic the cAMP-dependent DNA synthesis or the differentiated phenotype in thyroid cells (37).

In this study, we examined whether TSH and cAMP activated p38-MAPK using CHO cells stably transfected with the human TSH receptor (hTSHR-CHO) (38) and the thyroid FRTL-5 cell line. The latter retains most of the features of differentiated thyroid follicular cells, such as TSH-dependent growth and expression of differentiation markers. We find that TSH stimulates p38-MAPK activity in hTSHR-CHO cells. This activation is reproduced by cAMP in hTSHR-CHO cells and in FRTL-5 cells. Inhibition of the p38-MAPK pathway attenuates cAMP-induced expression of NIS. The activation of p38-MAPK is PKA-dependent and involves the small GTPase Rac1. TSH and cAMP also cause the activation of p38-MAPK through the generation of reactive oxygen species.

EXPERIMENTAL PROCEDURES

Materials—[γ-^{32}P]ATP (3000 Ci/mmol) was from PerkinElmer Life Sciences. LipofectAMINE PLUS reagent and Opti-MEM1 were from Life Technologies, Inc. Coon's modified Ham's F-12 medium was from Seromed. Anti-phospho-p38-MAPK (Thr^{183}/Tyr^{185}), anti-phospho-MKK3/MKK6 (Ser^{189}/Ser^{207}), anti-phospho-CREB (Ser^{133}) and anti-CREB polyclonal antibodies were obtained from New England Biolabs, Inc. (Beverly, MA). p38-MAPK (C20) antibodies were purchased from Santa Cruz Biotechnology Inc. Monoclonal anti-HA (12CA5) antibody was from Berkeley Company, Inc. MAPKAP kinase-2 immunoprecipitation kinase assay kit was from Upstate Biotechnology Inc. H89, SB 203580, and bovine TSH were purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA). Wortmannin, rapamycin, and GF109203X were from Biomol Research Laboratories, Inc. Recombinant human TSH (rhTSH) was from Genzyme Diagnostics. Diphenyleneiodonium (DPI) was synthesized as described previously (39).

pECE-HA-tagged p38α-MAPK was a generous gift from Dr. J. Pouysségur (UMR134, CNRS Nice, France). pEXV-Myc-tagged Rac N17 was kindly provided by Dr. A. Hall (Department of Biochemistry and Molecular Biology, University College London, London, UK). pcDNA3-HA-tagged Ras N17 was a gift from Dr. A. Eychène (UMR 146, CNRS Orsay, France).

Cell Culture, Transient Transfections, and Extracts—hTSHR-CHO cells were a generous gift from Dr. G. Vassart (Université Libre de Bruxelles, Belgium). They were grown to subconfluence in Ham's F-12 medium supplemented with 1 mM pyruvate and 10% heat-inactivated fetal calf serum (FCS) in presence of geneticin. Serum was withdrawn from subconfluent cultures 48 h before stimulation with rhTSH. Wild-type CHO cells were cultured in the same conditions without geneticin. FRTL-5 cells (American Type Culture Collection CRL 8305) were grown in Coon's modified Ham's F-12 medium supplemented with 5% heat-inactivated FCS, bovine TSH (1 milliunit/ml), insulin (10 μg/ml), and transferrin (5 μg/ml). Serum and hormones were withdrawn from subconfluent cultures 48 h before stimulation. ER22 cells derived from CCL39 cells by stable expression of the EGF receptor were grown as described previously (40).

The intracellular cAMP accumulation was determined according to Evans (41). Transient transfections were performed using LipofectAMINE PLUS reagent. Cells were incubated for 24 h in FCS medium after transfection and deprived of FCS for an additional 24 h before stimulation. Cell extracts were prepared in buffer A containing 80 mM β-glycerophosphate, pH 7.4, 20 mM EGTA, 15 mM $MgCl_2$, and a mixture of protease and phosphatase inhibitors (42). For immunoblotting with phospho-CREB or CREB antibodies, total cell lysates were prepared by scraping the cells into Laemmli sample buffer.

Western Blot Analysis and Immunoprecipitation—Proteins were resolved on 12% SDS-PAGE and transferred onto nitrocellulose membranes, and the membranes were blocked with 3% bovine serum albumin. The blots were probed overnight with the appropriate antibody. Antibody binding was revealed by ECL or by the alkaline phosphatase system.

p38α-MAPK, HA-tagged p38α-MAPK and MAPKAP kinase-2 were immunoprecipitated from cell extracts containing 1% Triton X-100 by incubation for 2 h at 4 °C with 2 μg of polyclonal anti-p38-MAPK antibody (C20) or 5 μg of monoclonal anti-HA antibody or 3.6 μg of polyclonal anti-MAPKAP kinase-2 antibody, followed by an additional 1 h with protein G-Sepharose. The precipitates were washed twice with buffer A containing 1% Triton and three times with the appropriate kinase assay buffer (see below).

RNA Analysis—RNA was extracted by the method of Chomczynski and Sacchi (43) and analyzed by Northern blot as described previously (44). Blots were hybridized with probes for NIS and GAPDH. The rat NIS cDNA probe was isolated by reverse transcription-polymerase chain reaction with the following primers: sense (5′-CCTTCTGGACTTTCATAGTGGG-3′) and antisense (5′-TGGGACCAGTAAGGTAGCTGAT-3′) and cloned in pCR 2.1 TOPO vector (Invitrogen BV, Groningen, The Netherlands).

In Vitro Kinase Assays—The NH_2-terminal domain of ATF2[1–109] was expressed as a glutathione *S*-transferase (GST) fusion protein using the pGEX-2T vector (Amersham Pharmacia Biotech), purified, and used as a substrate. p38α-MAPK or HA-tagged p38-MAPK were assayed by incubating immunoprecipitates with GST-ATF2[1–109] for 20 min at 30 °C in kinase assay buffer containing 20 mM HEPES, pH 7.4, 5 mM $MgCl_2$, 2 mM EGTA, 2 mM dithiothreitol, 1 mM Na_3VO_4, plus 40 μM ATP, and 5 μCi of [γ-^{32}P]ATP. The reactions were stopped by adding Laemmli sample buffer. Phosphorylated products were analyzed by 12% SDS-PAGE and autoradiography. The radioactivity incorporated into GST-ATF2 was counted by liquid scintillation.

MAPKAP kinase-2 was assayed by incubating immunoprecipitates with 0.125 mM of synthetic peptide (KKLNRTLSVA) substrate for 30 min at 30 °C in kinase assay buffer containing 20 mM MOPS, pH 7.2, 9 mM $MgCl_2$, 5 mM EGTA, 25 mM β-glycerophosphate, 1 mM dithiothreitol, 1 mM Na_3VO_4, plus 60 μM ATP, and 5 μCi of [γ-^{32}P]ATP. The reactions were stopped by centrifugation, and 25-μl aliquots of the supernatant were spotted on p81 phosphocellulose squares. These were washed in 0.75% H_3PO_4, and the radioactivity was counted by liquid scintillation.

RESULTS

TSH-stimulated Phosphorylation of p38-MAPK in a TSH Receptor-dependent Manner—The intracellular production of cAMP was measured as a function of rhTSH concentration in hTSHR-CHO and wild-type CHO cells. As expected, rhTSH stimulated cAMP production (20-fold) in cells expressing TSH receptors in a dose-dependent manner (ED_{50} = ~0.1 milliunit/ml), but not in wild-type CHO cells (not shown).

The involvement of p38-MAPK in the response to TSH was determined by incubating hTSHR-CHO cells and wild-type CHO cells with rhTSH. The phosphorylation of p38-MAPK was analyzed as a function of incubation time by immunoblotting using a specific anti-phospho-p38-MAPK antibody. rhTSH (1 milliunit/ml) stimulated the phosphorylation of p38-MAPK in a time-dependent manner in hTSHR-CHO cells (Fig. 1*A*) but not in wild-type CHO cells (Fig. 1*B*). p38-MAPK phosphorylation occurred after incubation for 20 min and reached a maximum at 60–90 min. The activator of stress-activated protein kinases, anisomycin, stimulated p38-MAPK phosphorylation in both cell types. The same nitrocellulose membranes were probed with an antibody for p38α-MAPK (Fig. 1, *A* and *B*, *lower panels*), showing that the amount of p38α-MAPK remained constant with time. Fig. 1*C* shows that incubation of hTSHR-CHO cells for 60 min with increasing concentrations of rhTSH resulted in a dose-dependent phosphorylation of p38-MAPK (ED_{50} = 0.2 milliunit/ml).

Involvement of cAMP in the Stimulation of p38-MAPK Phosphorylation—When hTSHR-CHO cells were incubated with 10

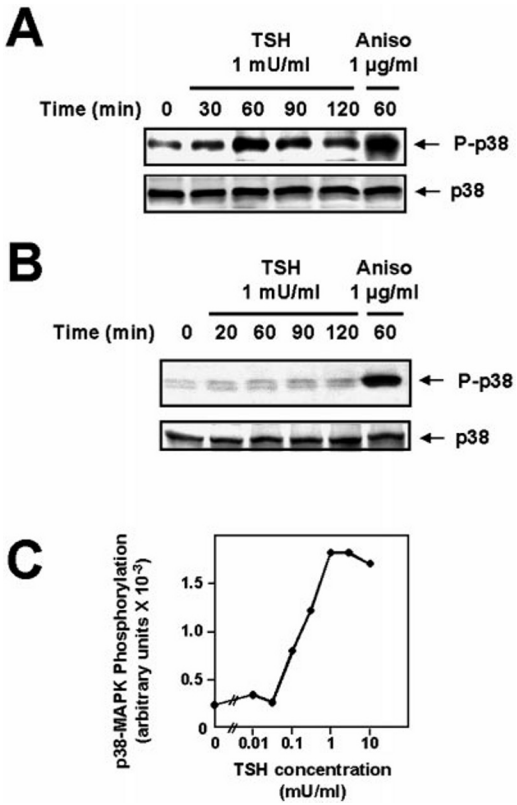

FIG. 1. **Effect of TSH on p38-MAPK phosphorylation.** *A* and *B*, time course of TSH-stimulated p38-MAPK phosphorylation in hTSHR-CHO cells (*A*) and wild-type CHO cells (*B*). Quiescent cells were treated with 1 milliunit/ml rhTSH or 1 μg/ml anisomycin (Aniso) for the indicated times. Cell extracts (30 μg) were separated by 12% SDS-PAGE, transferred to nitrocellulose membranes, and probed with anti-phospho-p38-MAPK antibody. Specific binding was detected by enhanced chemiluminescence (*upper panels*). Blots were stripped and reprobed with antibodies against unphosphorylated p38α-MAPK as a measure of protein loading (*lower panels*). *C*, dose-dependent activation of p38-MAPK by TSH in hTSHR-CHO cells. Cells were treated with increasing concentrations of rhTSH (0.01–10 milliunits/ml) for 60 min, and cell extracts were analyzed for p38-MAPK phosphorylation. Bands were quantified by densitometry using NIH image 1.60. The data shown are representative of three independent experiments.

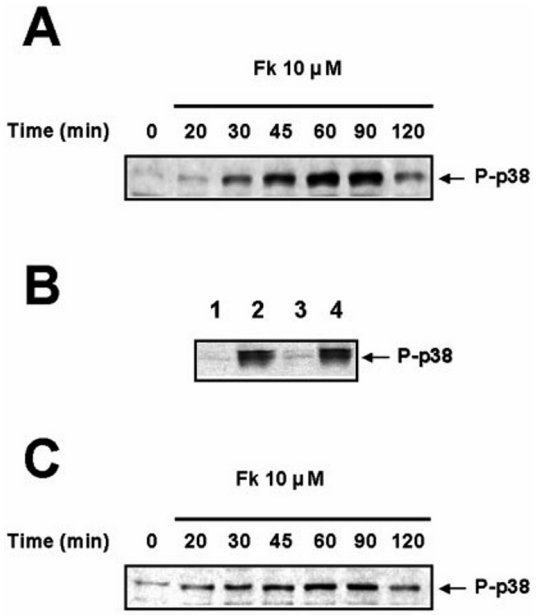

FIG. 2. **Implication of the cAMP signaling pathway in phosphorylation of p38-MAPK.** *A*, hTSHR-CHO cells were incubated with 10 μM forskolin (Fk) for the indicated times, and 30 μg of cell extracts were analyzed by Western blotting using an anti-phospho-p38-MAPK antibody. B, hTSHR-CHO cells were untreated (*lane 1*) or incubated with 10 μM Fk (*lane 2*), 10 μM dideoxyforskolin (*lane 3*), or 0.5 mM 8-bromo-cAMP (*lane 4*) for 60 min, and p38-MAPK phosphorylation was detected as above. *C*, quiescent FRTL-5 cells were incubated with 10 μM Fk for the indicated times, and cell extracts (30 μg) were analyzed for phospho-p38-MAPK, as in *A*. Three separate experiments were performed with similar results.

μM forskolin, a direct activator of adenylyl cyclase, the phosphorylation of p38-MAPK was stimulated with a time course similar to that of TSH (Fig. 2*A*). Dideoxyforskolin (10 μM), a structural analog of forskolin that does not activate adenylyl cyclase, did not promote the phosphorylation of p38-MAPK. A permeant analog of cAMP, 8-bromo-cyclic AMP (0.5 mM), stimulated phosphorylation of p38-MAPK (Fig. 2*B*).

We also determined whether forskolin induced the phosphorylation of p38-MAPK in FRTL-5 cells. p38 MAPK phosphorylation was stimulated by forskolin (10 μM) in a time-dependent manner (Fig. 2*C*). The time course of stimulation was similar to that in hTSHR-CHO cells, with the maximum effect being obtained at 60 min.

Effect of GF109203X and H89 on TSH-induced Phosphorylation of p38-MAPK—hTSHR-CHO cells were incubated with 5 μM of the protein kinase C inhibitor GF109203X for 1 h and then stimulated with TSH. The inhibitor had no effect on the TSH-induced phosphorylation of p38-MAPK (not shown).

hTSHR-CHO cells were incubated with the PKA inhibitor H89 for 1 h and then stimulated with forskolin to determine whether PKA was involved in the cascade of p38-MAPK activation. p38-MAPK phosphorylation was inhibited by 10 and 20 μM H89 (Fig. 3*A*). CREB phosphorylation was also inhibited by the same concentrations of H89 (Fig. 3*B*). Blots were reprobed with antibodies against p38α-MAPK or CREB as a measure of protein loading (*lower panels*). The effect of H89 on EGF-induced phosphorylation of p38-MAPK was studied to check the specificity of H89. ER22 cells were chosen for this study because neither CHO cells nor FRTL-5 cells expressed EGF receptor. EGF (100 ng/ml) stimulated p38-MAPK phosphorylation by 2–3-fold, and H89 (5, 10 and 20 μM) did not inhibit this stimulation (not shown). Taken together, these results suggest that PKA is involved in the cAMP signaling pathway leading to p38-MAPK phosphorylation.

Activation of p38-MAPK in Response to TSH and Forskolin—We checked whether the phosphorylation of p38-MAPK resulted in an increase of its activity. hTSHR-CHO cells were incubated with rhTSH, forskolin, or anisomycin, the p38-MAPK was immunoprecipitated from cell extracts with an antibody against the α-isoform of p38-MAPK, and its activity was assayed using GST-ATF2 as a substrate (Fig. 4). The p38α-MAPK activity was increased 5-fold by rhTSH and 3-fold by forskolin. These increased kinase activities were similar to that obtained with anisomycin (5-fold). TSH stimulation was completely abolished by incubating the cells with SB203580.

Activation of MAPKAP Kinase-2 by TSH and Forskolin, but Not CREB Phosphorylation, Is Dependent on p38-MAPK—The activity of MAPKAP kinase-2, a substrate of p38-MAPK (14) was measured in hTSHR-CHO cells treated with TSH or forskolin. MAPKAP kinase-2 was immunoprecipitated from untreated cells and from cells treated with rhTSH, forskolin, or anisomycin, and its activity was assayed using a synthetic peptide. Stimulation with TSH or forskolin led to a ~5-fold increase in MAPKAP kinase-2 activity, similar to that obtained with anisomycin (Fig. 5). This stimulation is consistent with the activation of p38-MAPK obtained with TSH and forskolin. As expected, the MAPKAP kinase-2 activity stimulated by TSH was completely inhibited by incubating the cells with

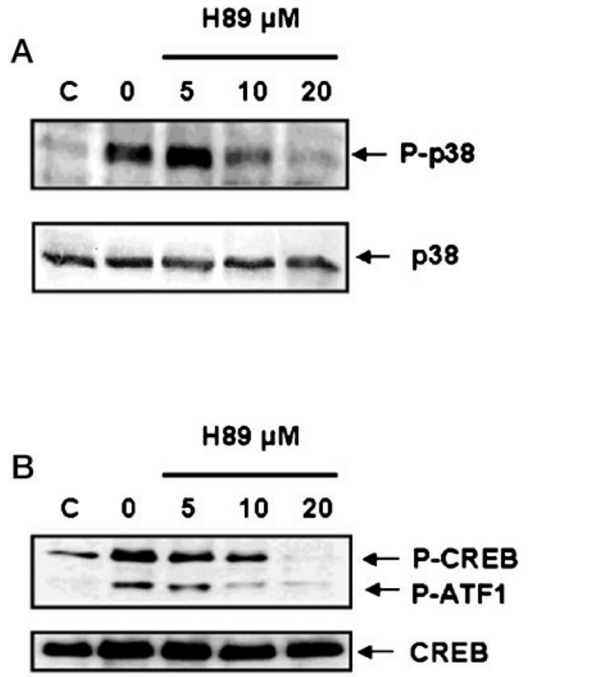

FIG. 3. **Effect of H89 on the phosphorylation of p38-MAPK and CREB.** *A*, hTSHR-CHO cells were incubated with 5–20 μM H89 for 60 min and then with 10 μM Fk for 60 min. Cell extracts (30 μg) were analyzed using anti-phospho-p38-MAPK antibody (*upper panel*). *B*, a similar experiment was performed as in *A*, but 50 μl of lysates were analyzed with anti-phospho-CREB antibody (*upper panel*). Blots were stripped and reprobed with antibodies against unphosphorylated p38α-MAPK or CREB (*lower panels*). The results are representative of at least two independent experiments.

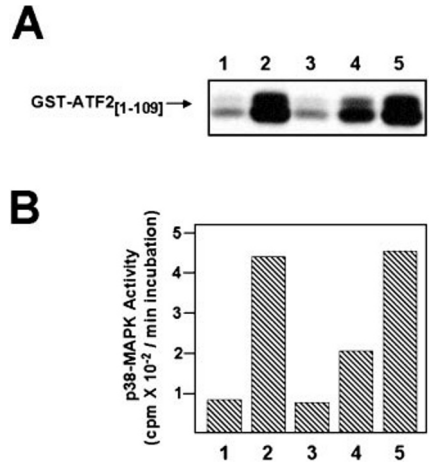

FIG. 4. **Activity of p38-MAPK stimulated by TSH, Fk, and anisomycin.** hTSHR-CHO cells were untreated (*lane 1*) or treated for 1 h with 1 milliunit/ml rhTSH (*lane 2*), 20 μM SB203580 for 1 h followed by 1 milliunit/ml rhTSH for 1 h (*lane 3*), 10 μM Fk (*lane 4*), or 1 μg/ml anisomycin (*lane 5*). *A*, cell lysate proteins (200 μg) were immunoprecipitated with anti-p38α-MAPK antibody, and the kinase was assayed *in vitro*. *B*, the GST-ATF2 band was excised, and the ^{32}P was incorporated into GST-ATF2 counted and expressed as cpm incorporated per min of incubation.

SB203580, confirming that the stimulation of MAPKAP kinase-2 activity was p38-MAPK-mediated.

It has been reported that MAPKAP kinase-2 phosphorylates CREB in response to fibroblast growth factor in neuroblastoma cells (45). Therefore, we investigated whether p38-MAPK was involved in TSH- or forskolin-induced CREB phosphorylation in hTSHR-CHO cells and in FRTL-5 cells. Cells were treated with forskolin in the presence and absence of SB203580, and

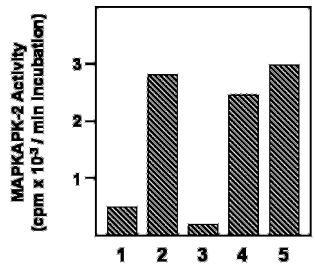

FIG. 5. **Correlation of p38-MAPK activation with MAPKAP kinase-2 activation by TSH or FK.** hTSHR-CHO cells were untreated (*column 1*) or treated for 1 h with: 1 milliunit/ml rhTSH (*column 2*), 20 μM SB203580 for 1 h followed by 1 milliunit/ml rhTSH for 1 h (*column 3*), 10 μM Fk (*column 4*), or 1 μg/ml anisomycin (*column 5*). MAPKAP kinase-2 was immunoprecipitated from 200 μg of cell lysates, and activity was assayed using a synthetic peptide. ^{32}P incorporated into the peptide was counted and expressed as cpm of ^{32}P incorporated per min of incubation. The results are representative of two independent experiments.

TABLE I

SB203580 has no effect on Fk-stimulated phosphorylation of CREB

hTSHR-CHO cells and FRTL-5 cells were treated (+SB) or not (−SB) with 20 μM SB203580 for 30 min, and then cells were treated with 10 μM forskolin for the indicated times. Cell extracts containing equal amounts of proteins were analyzed by SDS-PAGE and immunoblotted with an anti-phospho-CREB. The membranes were reprobed with CREB antibody. Quantification of specific bands was done by scanning densitometry using NIH Image 1.60. The ratio of phosphorylated CREB over the nonphosphorylated form was calculated, and the value for the untreated cells was taken as 1. The results are the means ± S.D. The number of independent experiments is given in parentheses.

Time	P-CREB/CREB			
	hTSHR-CHO cells		FRTL-5 cells	
	−SB	+SB	−SB	+SB
(min)				
10	2.24 (1)	2.08 (1)	1.86 ± 0.45 (6)	1.81 ± 0.54 (6)
20	2.11 ± 0.68 (5)	2.61 ± 0.64 (5)	1.95 ± 0.57 (6)	1.98 ± 0.60 (6)
60	2.38 ± 0.74 (6)	2.42 ± 0.73 (6)	1.61 ± 0.39 (5)	1.60 ± 0.46 (5)

CREB phosphorylation was analyzed by immunoblotting using a specific anti-phospho-CREB antibody. Table I shows that the inhibitor was without significant effect on forskolin-induced CREB phosphorylation in both cell types. Similar results were obtained with hTSHR-CHO cells stimulated by TSH (not shown).

Upstream Activators Involved in Stimulation of p38-MAPK by TSH or Forskolin—We examined the possible involvement of phosphatidylinositol 3-kinase (PI3K) or/and p70^{S6k} in TSH-induced p38-MAPK phosphorylation. hTSHR-CHO cells were incubated with 100 nM wortmannin for 30 min and then incubated with rhTSH. The drug had no effect on TSH-induced p38-MAPK phosphorylation (not shown). Incubation of hTSHR-CHO cells with 20 ng/ml rapamycin for 30 min had no effect on TSH-induced p38-MAPK phosphorylation (not shown).

We also examined whether TSH activated the kinases MKK3 and MKK6 using an antibody against their phosphorylated forms. rhTSH (1 milliunit/ml) stimulated the phosphorylation of MKK3 and/or MKK6 in a time-dependent manner, consistent with the time course of p38-MAPK activation (Fig. 6*A*).

To determine whether TSH and forskolin activated p38-MAPK by a mechanism implicating Rac1 or Ras, hTSHR-CHO cells and FRTL-5 cells were transfected with plasmids encoding HA-tagged p38-MAPK with or without plasmids encoding the dominant negative forms of Myc-tagged Rac1 (Rac1N17) or HA-tagged Ras (RasN17). HA-tagged p38-MAPK was immunoprecipitated using an anti-HA tag antibody, and p38-MAPK activity was assayed using GST-ATF2 as a substrate. Forskolin

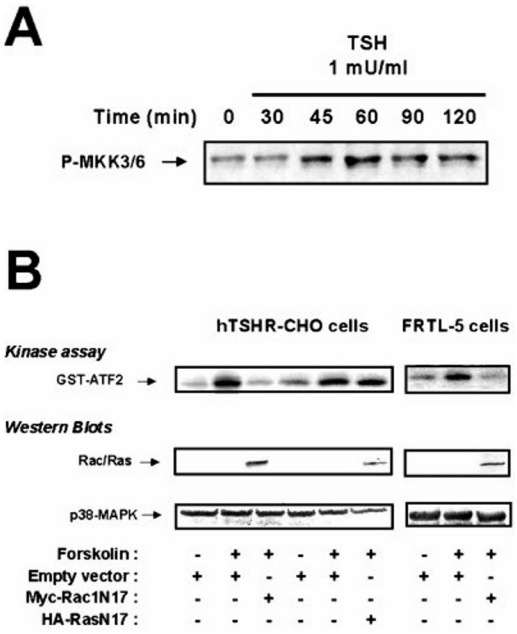

FIG. 6. **Upstream activators involved in stimulation of p38-MAPK by TSH or forskolin.** *A*, hTSHR-CHO cells were stimulated with 1 milliunit/ml TSH and equal amounts of total cell lysates (30 μg) were analyzed by Western blot using anti phospho-MKK3/MKK6 antibody as a function of time of TSH treatment. Results shown are representative of three independent experiments. *B*, hTSHR-CHO or FRTL-5 cells were cotransfected with 2.5 μg of plasmid expressing HA-tagged p38α-MAPK and with 2.5 μg of pEXV plasmid without a cDNA insert or with Myc-tagged Rac N17 cDNA. hTSHR-CHO cells were also cotransfected with 2.5 μg of plasmid expressing HA-tagged p38-MAPK and with 100 ng of pcDNA3 plasmid without a cDNA insert or with HA-tagged Ras N17 cDNA. Cells were incubated for 24 h after transfection, deprived of FCS for an additional 24 h, and treated with 10 μM Fk. Cell extracts (30 μg) were immunoprecipitated with anti-HA antibodies and assayed for p38-MAPK activity (kinase assay). The same cell extracts were analyzed by Western blot using anti-HA and anti-Myc antibodies to measure the expression of p38α-MAPK, Rac1, and Ras (Western blots).

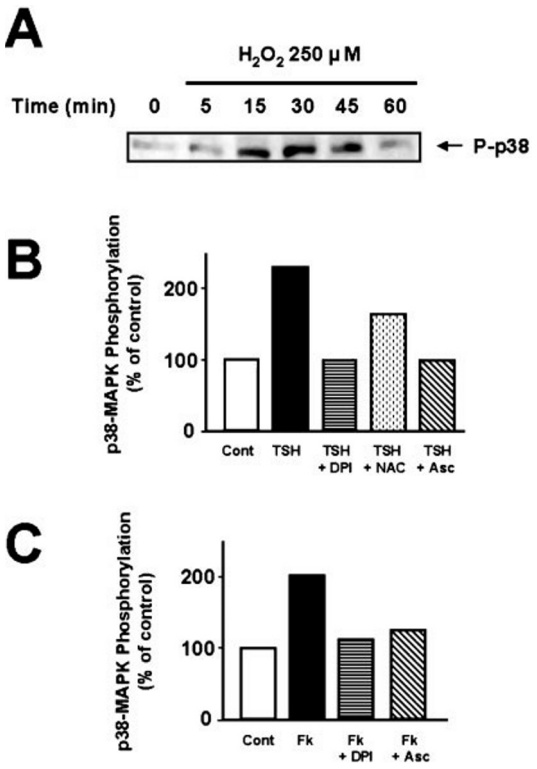

FIG. 7. **Involvement of ROS in the stimulation of p38-MAPK phosphorylation.** *A*, hTSHR-CHO cells were treated with 250 μM H_2O_2 for the indicated times. Cell extract proteins (30 μg) were analyzed by Western blot using an anti-phospho-p38-MAPK antibody. *B*, hTSHR-CHO cells were untreated, incubated with 1 milliunit/ml TSH for 60 min or preincubated with 2 μM of DPI for 30 min, with 20 mM *N*-acetylcysteine (*NAC*) for 30 min and with 100 μM ascorbic acid (*Asc*) for 2 h, before stimulation with TSH. *C*, FRTL-5 cells were untreated, incubated with 10 μM Fk for 60 min, or preincubated with 2 μM DPI for 30 min and with 100 μM ascorbic acid for 2 h, before stimulation with Fk. In *B* and *C*, cell extract proteins (30 μg) were analyzed by Western blot using an anti-phospho-p38-MAPK antibody. Bands were quantified by densitometry using NIH image 1.60.

stimulated HA-tagged p38-MAPK activity in hTSHR-CHO cells 2.7–3.5-fold, depending on the cotransfected empty vector (Fig. 6*B*). This stimulation was completely abolished by transfection with Rac1N17, whereas no significant effect was observed by transfection with RasN17. Rac1N17 inhibited also p38-MAPK activity in FRTL-5 cells. Western blots of the cell lysates were performed using anti-tag (HA or Myc) antibodies as a control for protein expression. These data indicate that Rac1 is involved in the pathway leading to p38-MAPK activation.

Involvement of Reactive Oxygen Species in TSH-induced Activation of p38-MAPK—We investigated the molecular mechanisms leading to activation of p38-MAPK by examining the role of reactive oxygen species (ROS) in its activation. hTSHR-CHO cells were treated with 250 μM H_2O_2, and the time course of p38-MAPK phosphorylation was followed to assess the effect of H_2O_2 on p38-MAPK. H_2O_2 stimulated p38-MAPK phosphorylation, which was maximum at 30 min (Fig. 7*A*). The H_2O_2-induced phosphorylation of p38-MAPK was dose-dependent, with the maximum effect at 250 μM (not shown). These data indicate that p38-MAPK in hTSHR-CHO cells can be regulated by a redox-sensitive mechanism.

Therefore, hTSHR-CHO cells were incubated with DPI, a potent inhibitor of flavonoid-containing enzymes, such as NADPH oxidase and nitric-oxide synthase, or with two antioxidants, *N*-acetylcysteine and ascorbic acid. The cells were then stimulated by TSH, and p38-MAPK phosphorylation was analyzed. DPI (2 μM) inhibited the phosphorylation of p38-MAPK induced by TSH (Fig. 7*B*). *N*-Acetylcysteine is an acidic compound that, when used as such (20 mM solution), completely inhibited p38-MAPK phosphorylation (not shown). But it had only a moderate effect (Fig. 7*B*) when the pH of the solution was brought to 7.4. In contrast, ascorbic acid (100 μM) completely inhibited the TSH-induced p38-MAPK phosphorylation. DPI also inhibited the forskolin-induced phosphorylation of p38-MAPK but had no effect on H_2O_2-stimulated p38-MAPK phosphorylation (not shown). In FRTL-5 cells, we observed the same inhibitory effect of DPI and ascorbic acid on forskolin-induced phosphorylation of p38-MAPK (Fig. 7*C*). Thus, the mechanism of activation of p38-MAPK by TSH and forskolin is likely to involve ROS.

Effect of p38-MAPK Inhibition on cAMP-induced Expression of NIS mRNA—Iodide is an essential component in the biosynthesis of thyroid hormones which is concentrated in the thyroid follicular cells by an active transporter, the NIS (46). NIS expression is known to be under the control of TSH in a cAMP-dependent manner (36). Indeed, treatment of FRTL-5 cells with forskolin (10 μM), followed by Northern blot analysis of NIS mRNA, resulted in an increase of a major 2.9-kilobase mRNA species, with a maximum effect at 24–36 h (not shown). To study the involvement of p38-MAPK pathway in this effect, FRTL-5 cells were stimulated by forskolin in the presence of SB203580 for 24 and 36 h. Control experiments were performed using SB202474, a chemical analog of SB203580 with no p38-MAPK inhibitory potency (7). As shown in Fig. 8*A*,

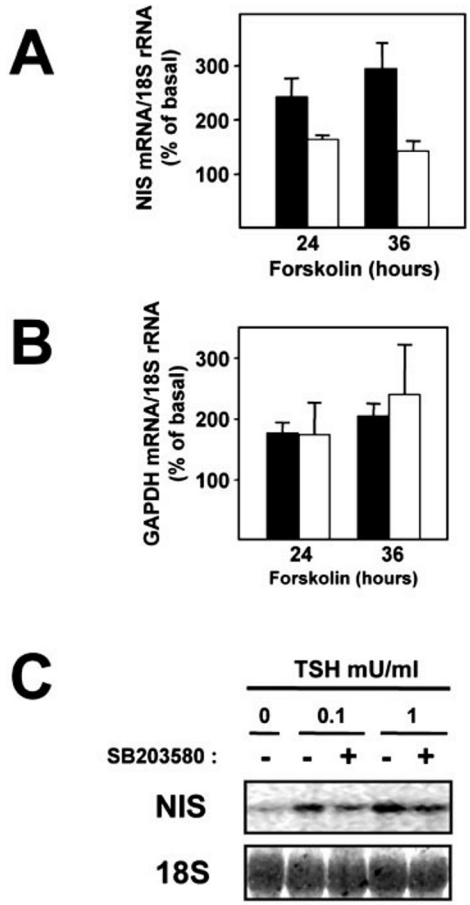

FIG. 8. **SB203580 inhibits the cAMP- and TSH-stimulated expression of NIS mRNA.** Confluent FRTL-5 cells were maintained in culture medium without TSH for 8 days. *A* and *B*, cells were treated with 20 μM SB203580 (*open bars*) or SB202474 (*black bars*) and, 1 h later, with 10 μM forskolin for an additional 24- or 36-h period. *C*, cells were treated or not with 20 μM SB203580 and, 1 h later, with the indicated concentrations of bovine TSH for an additional 48-h period. Total RNA was extracted from cells, separated by agarose gel electrophoresis, and blotted onto a nylon membrane. NIS (*A* and *C*) and GAPDH (*B*) transcripts were determined by hybridizing the membrane with labeled specific cDNA probes. Membranes were analyzed for NIS and GAPDH mRNA levels using an Instant-Imager (Packard, Instrument). 18 S ribosomal RNA bands were revealed by methylene blue staining and quantified by densitometry using NIH Image. The ratio of NIS or GAPDH mRNA radioactivity to the corresponding 18 S ribosomal RNA density was calculated and expressed as the percentage of basal values obtained in the absence of forskolin (*A* and *B*). Values are the means ± S.D. of three independent experiments.

SB203580 significantly inhibited forskolin-stimulated expression of NIS mRNA by 50–70%. In contrast, the inhibitor had no effect on GAPDH mRNA levels (Fig. 8*B*). SB203580 also inhibited the TSH-stimulated expression of NIS mRNA (Fig. 8*C*).These results strongly suggest that the cAMP-dependent p38-MAPK pathway is involved in the regulation of NIS expression expression of NIS.

DISCUSSION

We have investigated the early steps of TSH signal transduction and found that TSH stimulates p38-MAPK phosphorylation and activity in CHO cells stably transfected with the human TSH receptor. TSH does not stimulate the phosphorylation of p38-MAPKs in wild-type CHO cells, indicating that the process is mediated by the TSH receptor. p38-MAPK appears to be the only MAPK whose phosphorylation is activated by TSH. Indeed, we have shown that TSH does not stimulate the phosphorylation of JNKs in hTSHR-CHO cells or in FRTL-5 cells and that TSH preparations could contain factor(s) responsible for activation of the ERK signaling pathway by a TSH receptor-independent mechanism (47).

The phosphorylation of p38-MAPK in hTSHR-CHO cells reached a maximum with 1 milliunit/ml of rhTSH, corresponding to the concentration that gave maximum stimulation of intracellular cAMP production. Moreover, treatment of hTSHR-CHO cells with forskolin or with bromo-cAMP reproduced the TSH effects. Thus, the phosphorylation of p38-MAPK is activated by a cAMP-dependent mechanism. Forskolin-dependent p38-MAPK phosphorylation also occurs in FRTL-5 thyroid cells with a time course similar to that in hTSHR-CHO cells, suggesting that this pathway operates in the thyroid gland. Recent studies indicate that cAMP is also involved in the activation of p38-MAPK by G protein-coupled receptors such as the dopamine receptor in neuroblastoma cells (48) and the FSH receptor in granulosa cells (49). Importantly, the p38-MAPK inhibitor SB203580 inhibits cAMP-induced expression of NIS mRNA, suggesting that the p38-MAPK pathway is likely to be involved in cAMP-induced expression of NIS mRNA. This is an important aspect of thyroid physiology, because NIS mediates active transport of iodide, and its function is crucial in the biosynthesis of thyroid hormones.

It was reported that PKA activity is required for both TSH-stimulated synthesis of DNA and thyroglobulin (50). We show that 10 μM of the PKA inhibitor H89 specifically inhibits forskolin-induced p38-MAPK phosphorylation. This concentration of H89 does not inhibit EGF-induced p38-MAPK phosphorylation, whereas it blocks CREB/ATF1 phosphorylation. However, we cannot exclude the involvement of another cAMP-dependent pathway such as that implicating the small G protein Rap1 (51). Cass *et al.* (52) showed that PKA is implicated in the activation of $p70^{S6k}$ by TSH and 8-bromo-cAMP in WRT cells. In our study, pretreatment of hTSHR-CHO cells with rapamycin showed that $p70^{S6k}$ is not involved in p38-MAPK activation by TSH. However, it is possible that p38-MAPK is involved in $p70^{S6k}$ activation, as during C2C12 muscle cell differentiation (53). We show that p38-MAPK was not regulated by a PI3K-dependent mechanism, and we have found no rapid activation of PI3Kα/β by TSH, either in hTSHR-CHO cells or in FRTL-5 cells or in primary cultures of porcine thyroid cells.[2]

In the thyroid gland, α and δ isoforms of p38-MAPKs are expressed with a predominance of the α isoform of p38-MAPKs, whereas the expression of the β and γ isoforms is very low (10). In our work, at least the α isoform of p38-MAPKs is activated by TSH and forskolin, as shown by immunoprecipitation with a specific antibody followed by kinase assay using GST-ATF2 as a substrate. TSH and forskolin stimulate also the activity of MAPKAP kinase-2, a kinase that lies immediately downstream of p38-MAPKs.

As expected, p38-MAPK activity was inhibited by SB203580. This inhibitor was reported to be highly specific for p38-MAPK both *in vitro* and *in vivo* and, even at concentrations as high as 100 μM, was found to have no effect on the activity of many other protein kinases (54). However, it has been recently reported that SB203580 could also inhibit the PI3K/PKB pathway (55). In the present study, we have shown that TSH-dependent p38-MAPK activity is not dependent on this pathway because inhibition of PI3K and of $p70^{S6k}$ had no effect, as detailed above. It has been also reported that SB203580 inhibits arachidonic acid metabolism in platelets and cyclooxygenase activities *in vitro* (56). Although TSH stimulates arachidonic acid release in FRTL-5 cells, this effect involves a pertussis toxin-sensitive G protein and is not cAMP mediated,

[2] M. Pomerance, unpublished observations.

whereas TSH alone has no effect on cyclooxygenase activity (57).

In neuroblastoma cells stimulated by fibroblast growth factor, MAPKAP kinase-2 has been reported to induce CREB phosphorylation, which is prevented by SB203580 (45). In hTSHR-CHO cells or in FRTL-5 cells, we show that SB203580 has no significant effect on CREB phosphorylation, suggesting that the SB203580-sensitive cAMP stimulation of NIS mRNA expression does not depend on CREB activation. MAPKAP kinase-2 can phosphorylate the small heat shock protein HSP27, which can modulate actin filament dynamics (58, 59). However, it remains to be established whether this pathway operates in thyroid cells.

The p38-MAPKs are probably activated by the upstream kinases MKK3/MKK6 in hTSHR-CHO cells, because they are phosphorylated by TSH with a time course similar to that of p38-MAPKs. It has been reported that the activation of p38-MAPK is regulated by Rac1 in response to inflammatory mediators (23) or by Ras in response to platelet-derived growth factor (60). Our study also shows that Rac1 is an upstream mediator in p38-MAPK activation by TSH and cAMP, whereas Ras does not appear to be involved. STE20-related protein kinases have been described to be implicated in the p38 MAPK pathway. Recently, new kinases belonging to this family were identified: one is regulated by PKA (61), and another is activated by oxidative stress (62), but they do not activate the p38-MAPK pathway. Therefore, the nature of the MAPKKKK(s) and MAPKKK(s) situated downstream of Rac1 and upstream to MKK3/MKK6 remains to be determined in thyroid cells.

The G protein-coupled angiotensin II receptor can activate p38-MAPK by a mechanism that involves ROS (63). Moreover, Bjorkman and Ekholm (64) reported the TSH-stimulated generation of H_2O_2 in FRTL-5 cells within 45 min. In the present work, we show that DPI inhibited TSH- and forskolin-induced p38-MAPK phosphorylation in hTSHR-CHO cells and in FRTL-5 cells. The effect of DPI was specific, because it did not inhibit H_2O_2-induced p38-MAPK phosphorylation. This indicates a role for flavonoid-containing enzymes such as NADPH oxidase(s) and NO synthase(s) in TSH signaling. Although we confirm that H_2O_2 activates p38-MAPK phosphorylation, the nature of the ROS involved in TSH signaling is unknown, because the superoxide and NO radicals also appear to activate p38-MAPK (63, 65). Ascorbic acid is an effective free radical scavenger, whereas *N*-acetylcysteine replenishes intracellular reduced glutathione stores, which in turn are used by the H_2O_2-removing enzyme glutathione peroxidase (66). We find that ascorbic acid is more effective than *N*-acetylcysteine in inhibiting TSH-induced p38-MAPK phosphorylation. This suggests that free radical species, such as the superoxide radical, are the signaling species rather than H_2O_2 itself.

In conclusion, the present study shed a new light on the signaling mechanism of TSH and cAMP by providing evidence that they activate the cascade MKK3/MKK6, p38α-MAPK, and MAPKAP kinase-2 by a PKA-dependent mechanism implicating Rac1 and ROS. Although MAPKAP kinase-2 is a likely candidate, the downstream targets of p38-MAPKs remain to be identified in the thyroid gland. p38-MAPK is a component of the signaling pathway activated by TSH and cAMP in thyroid cells that plays a role in the expression of NIS.

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