Rapid stimulation of mitogen-activated protein kinase of rat liver by prolactin

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Intraperitoneal prolactin administration to female rats caused a rapid and transient stimulation of hepatic mitogen-activated kinase (MAP kinase) activity measured *in vitro* as cytosolic phosphotransferase capacity towards two specific substrates. Myelin basic protein kinase activity of MAP kinase immunoprecipitates confirmed the specificity and magnified the prolactin effect. Immunoblot experiments with anti-(MAP kinase) and anti-phosphotyrosine antibodies showed changes in both electrophoretic mobility and phosphotyrosine content of 40 and 44 kDa isoenzymes suggesting that prolactin affects these isoforms. Concomitant with the increase in MAP kinase activity, prolactin induced tyrosine phosphorylation in a number of liver proteins, suggesting a rapid involvement of tyrosine kinases which might be correlated in some way with MAP kinase activation. Protein kinase C activity, which has been implicated in the regulation of MAP kinase and in mediating the prolactin effect, does not seem to participate in MAP kinase activation.

INTRODUCTION

In addition to the classic effects on mammary gland [1] the pituitary peptide hormone prolactin is involved in a broad spectrum of physiological processes, including differentiation and proliferation, in a variety of tissues [2,3]. Prolactin effects are initiated by interaction with a specific receptor, which belongs to a large family of transmembrane proteins, including the receptors for growth hormone, erythropoietin, interleukin 2, 3, 4 and 6 and granulocyte–macrophage colony-stimulating factor [4–6]. A high concentration of prolactin receptors has been demonstrated in rat liver [5]. Acute administration of prolactin to rats elevates hepatic DNA synthesis, and chronic treatment causes hepatic hypertrophy [7]. The intracellular domain of the prolactin receptor does not possess any consensus sequence for intrinsic tyrosine kinase activity and ATP binding [5]. Although activation of protein kinase C (PKC) seems to play a role in the hepatic effects of prolactin [7], the actual signal-transduction pathway remains largely unknown.

In the present work we have investigated whether prolactin treatment stimulates mitogen-activated protein (MAP) kinase, a key component of the signalling pathway responsive to a wide range of mitogens and extracellular stimuli in different cell types [8,9]. Possible relationships between MAP kinase and other kinases were also studied.

MATERIALS AND METHODS

Materials

Ovine prolactin was obtained from Fluka (Buchs, Switzerland); leupeptin, sodium molybdate, sodium orthovanadate, phenylmethanesulphonyl fluoride, β-glycerophosphate, myelin basic protein (MBP), histone H1, Protein A–Sepharose and preimmune rabbit serum were obtained from Sigma (St. Louis, MO, U.S.A.); poly(vinylidene difluoride) (PVDF) membranes were obtained from Millipore Corp. (Bedford, MA, U.S.A.); [γ-^{32}P]ATP, biotinylated anti-rabbit IgG, streptavidin, biotinylated horseradish peroxidase (HRP) complex and the Enhanced chemiluminescence (ECL) Western-blotting detection kit were purchased from Amersham International (Amersham, Bucks., U.K.); MAP kinase substrate peptide (sequence APRTPGGRR, which includes amino acids 95–98 of bovine MBP), polyclonal anti-(rat MAP kinase R2) antibody (generated against a synthetic peptide with a sequence based on residues 333–367 of the C-terminus of the rat 43 kDa erk1 MAP kinase), monoclonal anti-phosphotyrosine cross-linked to HRP (clone 4G10) antibody were obtained from Upstate Biotechnology Inc. (Lake Placid, NY, U.S.A.). All other reagents were of analytical grade and purchased from BDH (Poole, Dorset, U.K.) or Sigma.

Animals and treatment

Female Wistar rats weighing 200 g from Nossan (Correzzana, Italy) were used. They were maintained with alternating 12 h cycles of light and dark and fed on a balanced diet *ad libitum*. Ovine prolactin was administered intraperitoneally at a dose of 22 mg/kg body weight [10], which causes an increase in hepatic DNA synthesis [11]. The animals were killed by decapitation (performed at 13:00 h) 5, 10, 20 and 30 min after prolactin injection, and the livers were rapidly excised.

MAP kinase activity

Livers were homogenized with 20 mM Tris/HCl, pH 7.4, containing 150 mM NaCl, 2 mM EGTA, 80 mM β-glycerophosphate, 1 mM phenylmethanesulphonyl fluoride, 1 mM sodium orthovanadate, 0.1 mM sodium molybdate, 0.1 % Triton X-100, 2 mM dithiothreitol, 20 μg/ml leupeptin and 0.1 mg/ml aprotinin. The samples were mixed briefly, kept on ice for 10 min and centrifuged at 100000 *g* for 60 min. Supernatants were assayed for MAP kinase activity as described by Campbell et al. [12] using two different substrates: MBP (10 μg) and MAP kinase substrate peptide [13,14] (25 μg).

Cytosolic proteins (15 μg) were incubated with or without substrate for 10 min at 30 °C in a reaction mixture containing 50 mM Tris/HCl, pH 7.4, 2 mM EGTA, 40 mM β-glycerophosphate, 12.5 mM $MgCl_2$, 100 μM ATP and 2 μCi of [γ-^{32}P]ATP. After incubation with MBP, samples were spotted on Whatman P-81 phosphocellulose paper; when MAP kinase

Abbreviations used: PKC, protein kinase C; MAP kinase, mitogen-activated protein kinase; MBP, myelin basic protein; HRP, horseradish peroxidase; PVDF, poly(vinylidene difluoride); ECL, enhanced chemiluminescence.

substrate peptide was used, 5% trichloroacetic acid and 100 μg of BSA were added; the samples were then centrifuged and portions of supernatant were spotted on P-81 paper.

The papers were washed several times with 180 mM H_3PO_4 to remove background radioactivity, rinsed with 95% ethanol, air-dried and counted for radioactivity in a liquid-scintillation counter. The kinase activity was calculated as the difference between ^{32}P incorporation in the presence and absence of substrate.

MBP kinase assay of MAP kinase immunoprecipitates

Liver cytosolic proteins (300 μg) in 500 μl of buffer containing 25 mM Tris/HCl (pH 8), 0.5 mM EGTA (pH 7.4), 137 mM NaCl, 1% Nonidet P-40, 1 mM sodium orthovanadate, 10 mM NaF and protease inhibitors were immunoprecipitated with 4 μg of anti-(MAP kinase R2) antibody or preimmune rabbit serum for mock precipitation. The mixture was rocked for 4 h at 4 °C. Then 100 μl of 10% resuspended Protein A–Sepharose was added to each sample and the mixture was rocked for 1 h at 4 °C. Immunoprecipitates were washed sequentially with the buffer, with 1 M NaCl, with 25 mM Tris/HCl, pH 7.4, and with kinase buffer without MBP, $MgCl_2$ and ATP. MBP kinase assay was carried out in 100 μl of complete kinase buffer containing 25 mM Tris/HCl (pH 7.4), 1 mM dithiothreitol, 25 mM β-glycerophosphate, 10 mM $MgCl_2$, 50 μM ATP, 25 μg MBP, protease inhibitors and 10 μCi of [γ-^{32}P]ATP at 30 °C for 10 min in a rotary incubator [15].

The beads were spun and two 20 μl portions of supernatant were spotted on P-81 phosphocellulose paper, washed and counted for radioactivity as described above. Radioactivity in the preimmune serum immunoprecipitate was subtracted from that in the MAP kinase immunoprecipitate [15]. Portions of supernatant were also analysed by SDS/PAGE. Gels were dried and exposed to Kodak X-Omat film for autoradiography.

Preparation of lysates from rat liver

Rat livers were dissolved (1:10, w/v) in boiling SDS lysis buffer [63 mM Tris/HCl, pH 6.8, containing 2% (w/v) SDS, 25% (v/v) glycerol, 100 mM sodium orthovanadate, 0.1 mM sodium molybdate, 100 μg/ml leupeptin and 100 μg/ml aprotinin] [12], boiled for 5 min, mixed vigorously, frozen and stored at −80 °C before electrophoresis. Protein was determined by the Bradford method [16].

Immunoblot analysis

Lysate or cytosolic protein (80–150 μg) was subjected to SDS/PAGE in 10% polyacrylamide gels [17] and transferred to PVDF membranes using a Bio-Rad Trans-Blot semi-dry cell [18]. The membranes were blocked with 3% BSA in 10 mM Tris/HCl pH 7.4, containing 150 mM NaCl (Tris-buffered saline) then probed with either monoclonal anti-phosphotyrosine HRP-conjugated antibody, diluted to 1 μg/ml in Tris-buffered saline containing 3% BSA, for 3 h or polyclonal anti-(rat MAP kinase R2) antibody, diluted to 0.5 μg/ml in Tris-buffered saline containing 0.1% BSA and 0.1% Tween-20, for 2 h. Anti-(MAP kinase) antibody was detected using biotinylated anti-species secondary antibody, streptavidin–biotinylated HRP complex. When the same blots were used for both determinations, the blots were stripped by incubation in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris/HCl, pH 6.7) for 1 h at 50 °C. After this procedure the blots were blocked with buffer containing 3% BSA and reprobed with a different antibody [19]. Immunoreactive proteins were made visible by the ECL method. Densitometric analysis was performed with an LKB Ultroscan laser densitometer.

Assay of PKC activity

Extracts of rat liver membranes were prepared as previously described [20]. The fractions were partially purified using DEAE-cellulose (DE-52) [21], and PKC activity was determined by an *in vitro* phosphorylation assay using histone H1 (type III) as substrate in the presence or absence of 500 μM $CaCl_2$ in excess of chelator concentration, 1 μg of diolein and 25 μg of phosphatidylserine. PKC assays were carried out at 30 °C for 10 min.

PKC activity was calculated by subtracting the basal activity from the values of kinase activity in the presence of $CaCl_2$ and phospholipids; data are expressed as pmol of ^{32}P transferred/10 min per mg of protein.

Statistical analysis

Significance of differences was tested by Student's t test.

RESULTS

The effects of prolactin administration were assessed by comparison with normal controls as rats injected with vehicle at various times of treatment were always in the normal range.

The involvement of MAP kinase activity in the signal-transmission mechanism of PRL in rat liver was studied by means of various procedures.

In vitro MAP kinase activity

The first series of experiments was designed to evaluate the phosphotransferase activity of crude cytosolic liver extracts towards MBP which is an excellent substrate for MAP kinases *in vitro* [8]. The activity was readily detectable in control animals and showed a significant increase (+66%) as early as 5 min after prolactin treatment (Table 1). Maximum activation occurred at 10 min (+100%), a slight decline in enzyme activity was observed at 20 min (+83%) and then the values returned to the normal range after 30 min.

The second series of experiments was performed with a more specific substrate for MAP kinase, a synthetic nonapeptide containing the threonine-phosphorylation site for the enzyme [13,14]. Under these experimental conditions, MAP kinase ac-

Table 1 Effect of *in vivo* prolactin treatment on MAP kinase activity of rat liver cytosol assayed using MBP and MAP kinase substrate peptide as substrates

Cytosolic proteins (15 μg) obtained from control and prolactin-treated rats were incubated with [γ-^{32}P]ATP in the presence of MBP or MAP kinase substrate peptide (containing amino acids 95–98 of bovine MBP) as described in the Materials and methods section. Data are means ± S.E.M. of at least five separate experiments. *$P < 0.05$ compared with control.

Time of treatment (min)	Phosphotransferase activity (pmol of ^{32}P/10 min per mg of protein)	
	MBP	MAP kinase substrate peptide
0	618 ± 93	90 ± 10
5	1024 ± 58*	148 ± 15*
10	1235 ± 150*	166 ± 16*
20	1131 ± 102*	142 ± 2*
30	562 ± 80	54 ± 10

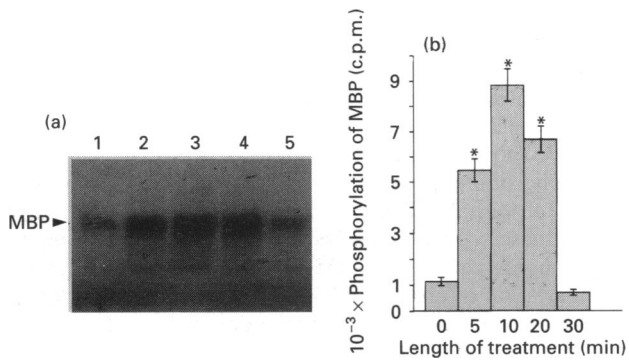


Figure 1 Phosphorylation of MBP by MAP kinase immunoprecipitates from cytosolic liver proteins

Cytosolic proteins (300 μg) were immunoprecipitated with 4 μg of polyclonal MAP kinase antibody. The immunoprecipitates were assayed for kinase activity toward MBP in the presence of [γ-^{32}P]ATP. After centrifugation, 20 μl of the assay mixture was subjected to SDS/PAGE followed by autoradiography and 20 μl was spotted on P-81 paper and counted. (**a**) MBP phosphorylation detected by autoradiography. Lane 1, control rats; lanes 2–5, rats treated with prolactin for 5, 10, 20 or 30 min. Comparable results were obtained in three separate experiments. (**b**) ^{32}P incorporated into MBP in c.p.m. (means ± S.E.M. of three separate experiments) calculated as described in the Materials and methods section. *Significantly different ($P < 0.01$) from control (0 min of treatment).

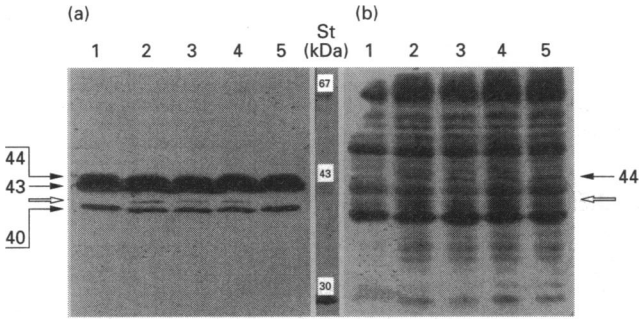


Figure 2 Immunoblot analysis of cytosolic proteins from rat liver with anti-(MAP kinase) (a) and anti-phosphotyrosine (b) antibodies

Cytosolic proteins (150 μg) were subjected to SDS/PAGE (10% gels) and transferred to PVDF membrane. The membrane was cut in half. The left half (**a**) was analysed with polyclonal anti-(MAP kinase), detected by biotinylated anti-rabbit IgG and streptavidin–HRP complex, and the right half (**b**) was probed with HRP-conjugated 4G10 anti-phosphotyrosine. Proteins were made visible by the ECL method and exposed to Kodak X-Omat films. Comparable results were obtained in four separate experiments. Lane 1, control rats; lanes 2–5, rats treated with prolactin for 5, 10, 20 or 30 min; St, molecular-mass standards. The arrows indicate 40, 43 and 44 kDa proteins; white arrows indicate a slight shift of part of the 40 kDa protein.

tivity showed different absolute values but the extent of the prolactin effect was very similar and its time course was superimposable on that of the former experiments (Table 1).

MBP phosphorylation assay by MAP kinase immunoprecipitates

To confirm the above results, the cytosolic liver proteins were subjected to immunoprecipitation with polyclonal anti-(MAP kinase R2) antibody followed by assay of kinase activity toward MBP. The immunoprecipitates from control animals were able to phosphorylate MBP and prolactin treatment stimulated this kinase activity (Figure 1a). By this procedure the extent of the prolactin effect was always greater than that seen when the kinase was assayed in crude extracts. The increase in ^{32}P in MBP was about five-, eight- and six-fold greater than that in controls after 5, 10 and 20 min of treatment respectively. The MBP kinase activity returned to the normal range 30 min after prolactin injection (Figure 1b).

Immunoblotting experiments

Multiple MAP kinase isoforms exist. They require phosphorylation in both tyrosine and threonine residues for maximal activation [8,22]. To identify the isoenzymes involved in prolactin-induced MAP kinase activation we examined the electrophoretic behaviour of MAP kinase proteins and their phosphotyrosine content by immunoblotting experiments. When cytosolic liver proteins from control rats were probed with the polyclonal anti-(MAP kinase) antibody, which recognizes the products of *erk1–erk2* and *mpk* genes, three bands were detected (Figure 2a): an abundant 43 kDa protein and two minor proteins of 44 and 40 kDa. In prolactin-treated rats a small fraction of the 40 kDa MAP kinase showed a lower electrophoretic mobility with respect to control animals which presumably reflects the increase in its phosphorylation, as observed also by others [23,24]. This slight shift occurred as soon as 5 min after treatment and was maintained for 20 min thereafter.

The same blots, after removal of anti-(MAP kinase) antibodies, or the other half of Western blots from identical samples, were then probed with an anti-phosphotyrosine monoclonal antibody. The phosphotyrosine signal was clearly detected in the upper region of the 40 kDa protein where the prolactin-induced shift occurs and in the correspondence of the 44 kDa band (Figure 2b).

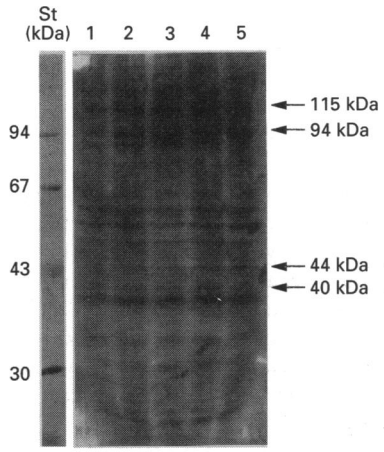
Kinase assays performed on renatured proteins in gels polymerized in the presence of MBP as substrate as described by Kameshita and Fujisawa [25] provided no better information than previous immunoblotting experiments. In crude cytosolic liver extracts from both control rats and those treated for 5 and 10 min with prolactin, ^{32}P was mostly incorporated into the 43–44 kDa region. Densitometric analysis demonstrates that this incorporation increases by 20% as early as 5 min after hormonal injection. In the 40 kDa region the amount of ^{32}P was smaller and it never displayed the upwards shift (results not shown).

PKC and tyrosine kinase activities

PKC has been implicated in the transmembrane signalling of the prolactin-induced stimulus to cell proliferation [26] and there is evidence for a PKC-dependent mechanism in the regulation of MAP kinase activity [27,28]; therefore we evaluated the time course of liver PKC activity after prolactin treatment. Investigations of *in vitro* PKC activity soon (5–10 min) after treatment revealed no changes, and a 10% non-significant increase was evident only 20 min after prolactin injection (4855 ± 64 pmol of ^{32}P incorporated into histone/10 min per mg of protein compared with a control value of 4363 ± 355 pmol/10 min per mg of protein). These observations exclude the involvement of PKC as a trigger of prolactin-induced MAP kinase activity, which occurs as early as 5 min after hormonal treatment.

Tyrosine kinase activity in cytosol and membrane preparations assayed by *in vitro* phosphorylation of the synthetic substrate poly(GluNaTyr) (4:1) [29] was the same in control and prolactin-treated rats (results not shown).

Analysis of tyrosine phosphorylation in liver proteins from control and prolactin-treated rats by anti-phosphotyrosine immunoblotting of whole cell lysates (Figure 3) confirmed the increase in phosphotyrosine in the 40 and 44 kDa MAP kinase proteins and showed an analogous prolactin effect on two protein bands with apparent molecular masses of 115 and 94 kDa. As early as 5 min after treatment, both proteins showed a doubling



Densitometric analysis

Proteins	94 kDa	115 kDa
Lane 1	1	1
Lane 2	2.1	2.3
Lane 3	2.1	2.4
Lane 4	2.3	1.9
Lane 5	2.5	2

Figure 3 Immunoblot analysis of lysates of rat liver with anti-phosphotyrosine antibody

Proteins (80 μg) (see the Materials and methods section) were resolved by SDS/PAGE (10% gels) and then transferred to PVDF membrane. The blot was probed with monoclonal HRP-conjugated anti-phosphotyrosine antibody detected by the ECL method. Lanes: St, molecular-mass standards; 1, control rats; 2–5, rats treated with prolactin for 5, 10, 20 or 30 min. The arrows indicate the 115 and 94 kDa proteins and the 44 and 40 kDa MAP kinase proteins. Densitometric analysis was performed on the 115 and 94 kDa proteins using an LKB Ultroscan laser densitometer. A_{663} of the control was taken as 1. Comparable results were obtained in four separate experiments.

of phosphotyrosine content which was maintained throughout the period of our observation (30 min) (Figure 3).

DISCUSSION

To our knowledge this is the first report showing that *in vivo* prolactin treatment can increase rat liver MAP kinase activity. The demonstration that prolactin injection stimulates enzyme activity as promptly as 5 min after injection with maximal activation after 10 min has been obtained by the use of different procedures and suggests that MAP kinase might be involved in triggering the effects of prolactin.

The results of immunoblot experiments indicate that two of the three enzyme isoforms detected in liver by anti-(MAP kinase) polyclonal antibody, i.e. the 40 and 44 kDa isoenzymes, are affected by the hormone. Although the gel-shift assay should be regarded with caution, our interpretation is also supported by the demonstration of a concomitant increase in phosphotyrosine content of the two isoforms and increased enzyme activity *in vitro*.

Our results are consistent with the hepatic mitogenic action of prolactin [30] and with the key role of MAP kinases in cell-cycle activation [8,9]: MAP kinase is responsible for the earliest events that occur in the release of cells from cell-cycle arrest, such as activation of transcriptional factors and downstream kinases [31].

It has been recently reported that growth hormone and granulocyte–macrophage colony-stimulating factor stimulate MAP kinase activity in 3T3 F442A fibroblasts and human neutrophils respectively [12,19]. The analogies between the receptors for these two agents and that of prolactin [4] can explain the similarity between these results and our own.

PKC appears to be involved in mediating prolactin action in several target tissues [23,32] including rat liver [10,30,33], and has been implicated in regulation of the MAP kinase cascade [27,28]. However, the fact that the membrane-bound PKC activity begins to increase only 20 min after prolactin injection seems to exclude a role for this enzyme in MAP kinase activation, which is seen as soon as 5 min after hormone treatment. Also the ability of growth hormone to stimulate MAP kinase in 3T3-F442A cells does not depend on PKC activation [34].

We have shown that prolactin induces a prompt increase in tyrosine phosphorylation of 115 and 94 kDa rat liver proteins concomitant with MAP kinase activation. Evidence for rapid tyrosine kinase activation has been reported in prolactin-treated Nb_2 lymphoma cells *in vitro* [35]: prolactin promotes tyrosine phosphorylation of proteins of similar size in Nb_2 cells [36] and rat liver cells. Quite recently the association of prolactin receptor with $p60^{src}$ [37] and the involvement of the Janus tyrosine kinase family in signal transduction of prolactin [38] have been reported. Thus, as demonstrated for growth-hormone and interleukin 2 receptors [39], a mechanism involving the association of prolactin receptor with other cellular proteins endowed with tyrosine kinase activity seems probable; the rapid tyrosine phosphorylation of a number of cellular proteins is in keeping with this possibility.

The pathways that regulate MAP kinase activity include a kinase cascade which might be activated by different mechanisms [22,40], and very recently a possible relationship between Janus kinase 2 and MAP kinase was proposed in a model for cytokine signalling pathways [41]. Additional investigations are needed to clarify whether and how prolactin-induced tyrosine kinase activity is relevant to MAP kinase activation in the liver. Studies are in progress to identify the upstream factors responsible for prolactin-induced MAP kinase activation.

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