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Activation of mitogen activated protein (MAP) kinases by vanadate is independent of insulin receptor autophosphorylation

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Abstract

Treatment of Chinese hamster ovary (CHO) cells over-expressing the human insulin receptor (CHO-HIRc) with the insulin mimetic agent, vanadate, resulted in a dose- and time-dependent tyrosine phosphorylation of two proteins with apparent molecular sizes of 42 kDa (p42) and 44 kDa (p44). However, vanadate was unable to stimulate the tyrosyl phosphorylation of the β-subunit of the insulin receptor. By using myelin basic protein (MBP) as the substrate to measure mitogen-activated protein (MAP) kinase activity in whole cell lysates, vanadate-stimulated tyrosyl phosphorylation of p42 and p44 was associated with a dose- and time-dependent activation of MAP kinase activity. Furthermore, affinity purification of cell lysates on anti-phosphotyrosine agarose column followed by immunoblotting with a specific antibody to MAP kinases demonstrated that vanadate treatment increased the tyrosyl phosphorylation of both $p44^{mapk}$ and $p42^{mapk}$ by several folds, as compared to controls, in concert with MAP kinase activation. In addition, retardation in gel mobility further confirmed that vanadate treatment increased the phosphorylation of $p44^{mapk}$ and $p42^{mapk}$ in CHO-HIRc. A similar effect of vanadate on MAP kinase tyrosyl phosphorylation and activation was also observed in CHO cells over-expressing a protein tyrosine kinase-deficient insulin receptor (CHO-1018). These results demonstrate that the protein tyrosine kinase activity of the insulin receptor may not be required in the signaling pathways leading to the vanadate-mediated tyrosyl phosphorylation and activation of MAP kinases.

Key words: Mitogen activated protein kinase; Protein tyrosyl phosphorylation; Insulin receptor β-subunit; Protein tyrosine phosphatase; Mutant insulin receptor

1. Introduction

The trace element, vanadate, has been shown to mimic several of the metabolic and growth promoting effects of insulin (reviewed in [1,2]). The most remarkable insulinomimetic effect of vanadate is its ability to normalise blood glucose levels in various animal models of insulin-dependent and non-insulin-dependent diabetes mellitus [3–7].

Insulin initiates its biological responses by binding to a specific plasma membrane receptor leading to the activation, by autophosphorylation, of an intrinsic protein tyrosine kinase (PTK) activity of the receptor β-subunit (reviewed in [8]). Insulin receptor autophosphorylation and activation of its PTK activity is believed to be a major pathway for mediating the pleiotropic effects of insulin [8,9]. However, the mechanism by which vanadate exerts its insulin-like effect remains controversial. Initially, the insulinomimetic effect of vanadate was implicated in its ability to stimulate insulin receptor PTK activity [10], but recent studies have suggested its action to be distal to the insulin receptor PTK activation [11–13].

A variety of external stimuli, including insulin, have been shown to stimulate a group of closely related protein serine/threonine kinases called mitogen activated protein kinases (MAPKs), also termed extracellular signal-regulated kinases (ERKs), two of which are known as $p44^{mapk/ERK1}$ and $p42^{mapk/ERK2}$ (reviewed in [14,15]). MAPKs/ERKs are activated by phosphorylation on tyrosine and threonine residues by an upstream kinase identified as a dual specificity MAP kinase kinase or ERK kinase (MEK) [16] and are believed to play a central role in insulin signal transduction [17]. Therefore, we undertook the present investigation to examine whether, similar to insulin, vanadate can also modulate the activation of MAPKs/ERKs and, if an active insulin receptor-protein tyrosine kinase is required in this process. In these studies we have used Chinese hamster ovary (CHO) cells over-expressing either a wild-type (CHO-HIRc) or a protein tyrosine kinase-deficient insulin receptor (CHO-1018). We demonstrate that vanadate mimics insulin in stimulating the tyrosyl phosphorylation and activation of two isoforms of MAPK/ERKs, $p44^{mapk}$ and $p42^{mapk}$. However, the vanadate-mediated effects were independent of insulin receptor autophosphorylation.

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2. Materials and methods

2.1. Materials

Parental CHO cells, CHO cells over-expressing a mutant human insulin receptor in which Lys^{1018} has been replaced by alanine causing a complete loss of the PTK activity of insulin receptor (CHO-1018), and CHO cells over-expressing a normal human insulin receptor (CHO-HIRc), were a kind gift from Dr. Morris F. White (Joslin Diabetes Centre, Boston, MA, USA). Insulin receptor antibody was generously provided by Dr. Barry I. Posner (Royal Victoria Hospital, McGill University, Montréal, Qué., Canada). Insulin was from Eli Lilly Co. (Indianapolis, IN, USA). Myelin basic protein (MBP) and sodium orthovanadate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Anti-phosphotyrosine antibody, anti-MAP kinase antibody (raised against a peptide based upon residues 333–367 of the C-terminus of the rat 43 kDa ERK1) that detects several isoforms of MAP kinases, including $p44^{mapk}$ and $p42^{mapk}$, and anti-phosphotyrosine affinity column were from Upstate Biotechnology (Lake Placid, NY, USA). Goat anti-mouse or anti-rabbit IgG conjugated to alkaline phosphatase were purchased from Bio-Rad (Mississauga, Ont., Canada).

2.2. Cell culture

CHO cells were maintained on HAM's F-12 medium containing 10% foetal bovine serum. Cells were grown to confluence in 100 mm plates and incubated in serum-free F-12 medium for 18 h prior to the experiment.

2.3. Detection of phosphotyrosine-containing proteins

Tyrosine phosphorylation of cellular proteins stimulated in the absence or presence of insulin or vanadate was assessed by immunoblotting using anti-phosphotyrosine antibodies. Cells were stimulated with various concentrations of insulin and vanadate for the indicated time periods. The cells were lysed on ice in 400 μl of buffer A (25 mM Tris-HCl, pH 7.5, 25 mM NaCl, 1 mM Na-vanadate, 10 mM Na-fluoride, 10 mM Na-pyrophosphate, 20 nM okadaic acid, 0.5 mM EGTA, 1 mM phenylmethylsulphonyl fluoride, 10 μg/ml aprotinin, 1% Triton X-100, and 0.1% SDS). The lysates were clarified by centrifugation for 10 min at 10,000 × g and the supernatant boiled in Laemmli's sample buffer [18]. The samples were then electrophoresed on 10% SDS-polyacrylamide gels [19], transferred to polyvinilidene difluoride (PVDF) membranes, blotted with anti-phosphotyrosine antibody (1:100) and detected using goat anti-mouse IgG conjugated to alkaline phosphatase (1:3,000).

2.4. Immunoaffinity purification of phosphotyrosyl proteins and MAP kinase immunoblotting

The cell lysates were affinity-purified on anti-phosphotyrosine-agarose column and immunoblotted using a MAP kinase antibody. For affinity purification of phosphotyrosyl proteins, briefly, the clarified cell lysates from control or stimulated cells were incubated for 30 min at 4°C with anti-phosphotyrosine agarose beads equilibrated in buffer B (20 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 10 mM EDTA, 0.2 mM Na-vanadate, 0.01% Na-azide, and 100 mM NaCl). The beads were washed 3 times with buffer B, and phosphotyrosine-containing proteins were eluted with 1 mM phosphotyrosine in buffer B. The eluates were boiled in 3× Laemmli's sample buffer, electrophoresed on 10% SDS-polyacrylamide gels, transferred to PVDF membranes, blotted with anti-MAP kinase antibody (1:500) and detected using goat anti-rabbit IgG conjugated to alkaline phosphatase. In the gel-shift experiments, MAP kinase immunoblotting was performed on whole cell lysates from control and stimulated CHO-HIRc cells.

2.5. MAP kinase assay

MAP kinase activity was determined using MBP as an exogenous substrate. To 5 μl of cell lysate (approximately 3–4 μg protein), 40 μl of kinase buffer (25 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM dithiothreitol, 40 μM ATP, 1 μM staurosporin, 15 μg MBP, 0.5 mM EGTA, and 0.2 μCi [γ^{32}P]ATP) was added. After 12 min. at 30°C, the reaction was stopped by adding 70 μl of 2× sample buffer. Samples were electrophoresed on 12% SDS-polyacrylamide mini gels, transferred on PVDF membranes and autoradiographed. In some experiments, the reaction was stopped by spotting 20 μl of the reaction mixture onto P-81 filter papers (2 × 2 cm), washed 4 times in 0.6% phosphoric acid and radioactivity counted.

2.6. Insulin receptor immunoprecipitation

CHO-HIRc cells were pre-labelled with [^{32}P]orthophosphate (0.2 mCi/ml) for 3 h. The cells were then stimulated for 5 min in the absence or presence of 100 nM insulin or 100 μM vanadate. The cells were washed 3 times with ice-cold PBS, frozen in liquid N_2 and lysed in buffer A. The lysate was centrifuged for 10 min at 10,000 × g and pre-cleared with protein A–Sepharose for 30 min. The pre-cleared lysate was incubated with the insulin receptor antibody for 4 h at 4°C and the immunoprecipitate was collected with protein A–Sepharose, washed 3 times with 50 mM HEPES buffer, pH 7.5, containing 0.1% Triton X-100 and 0.1% SDS, and 2 times with the above buffer without SDS. The immunoprecipitate was solubilized by boiling in sample buffer for 5 min. The solubilized fraction was electrophoresed on 10 SDS-polyacrylamide gels and transferred on PVDF membranes. PVDF membranes were treated with 1 M KOH for 2 h at 55°C to remove the phosphoserine/threonine [20] and autoradiographed [19].

3. Results

3.1. Effect of vanadate on protein tyrosine phosphorylation and MAP kinase activation

Treatment of CHO-HIRc cells with vanadate resulted in the tyrosyl phosphorylation of two major proteins corresponding to the molecular sizes of 44 kDa and 42 kDa (p44/p42) (Fig. 1A). The stimulation was concentration-dependent and was maximal at 0.1 mM (Fig. 1A). As expected, insulin stimulation of cells increased the tyrosyl phosphorylation of two more proteins of molecular sizes of 95 kDa and 185 kDa in addition to p44 and p42 (Fig. 1B). These two additional proteins with molecular sizes of 95 kDa and 185 kDa represent the insulin receptor β-subunit [21] and insulin receptor substrate-1 (IRS-1) [22], respectively. In vanadate-treated cells, however, there was no detectable phosphorylation of either the insulin receptor β-subunit or the IRS-1 even at 1 mM vanadate (Fig. 1A,B). Insulin-stimulated p44 and p42 phosphotyrosyl proteins have previously been identified as $p44^{mapk}$ and $p42^{mapk}$, respectively [14]. Since the molecular sizes of two MAP kinase isoforms, the tyrosyl phosphorylation and activation of which is stimulated by vanadate, are also 44 kDa and 42 kDa, it was of interest to investigate whether vanadate-mediated tyrosyl phosphorylation of these proteins is associated with an activation of the MAP kinase activity. As shown in Fig. 1C, treatment of CHO-HIRC cells with vanadate stimulated the MAP kinase activity, in a concentration dependent-manner, as judged by the increased phosphorylation of MBP. A significant MAP kinase activation was detectable at vanadate concentrations as low as 10 μM, which correlated with the degree of tyrosyl phosphorylation of p44 and p42 (Fig. 1A).

We next investigated the time–course of vanadate-mediated tyrosyl phosphorylation and MAP kinase activation in whole cell lysates. Exposure of CHO-HIRc cells with 100 μM vanadate resulted in a rapid and sustained tyrosyl phosphorylation of p44 and p42 (Fig. 2A). The increased phosphorylation was detectable as early as 1 min, reached a maximum at 2 min and remained un-

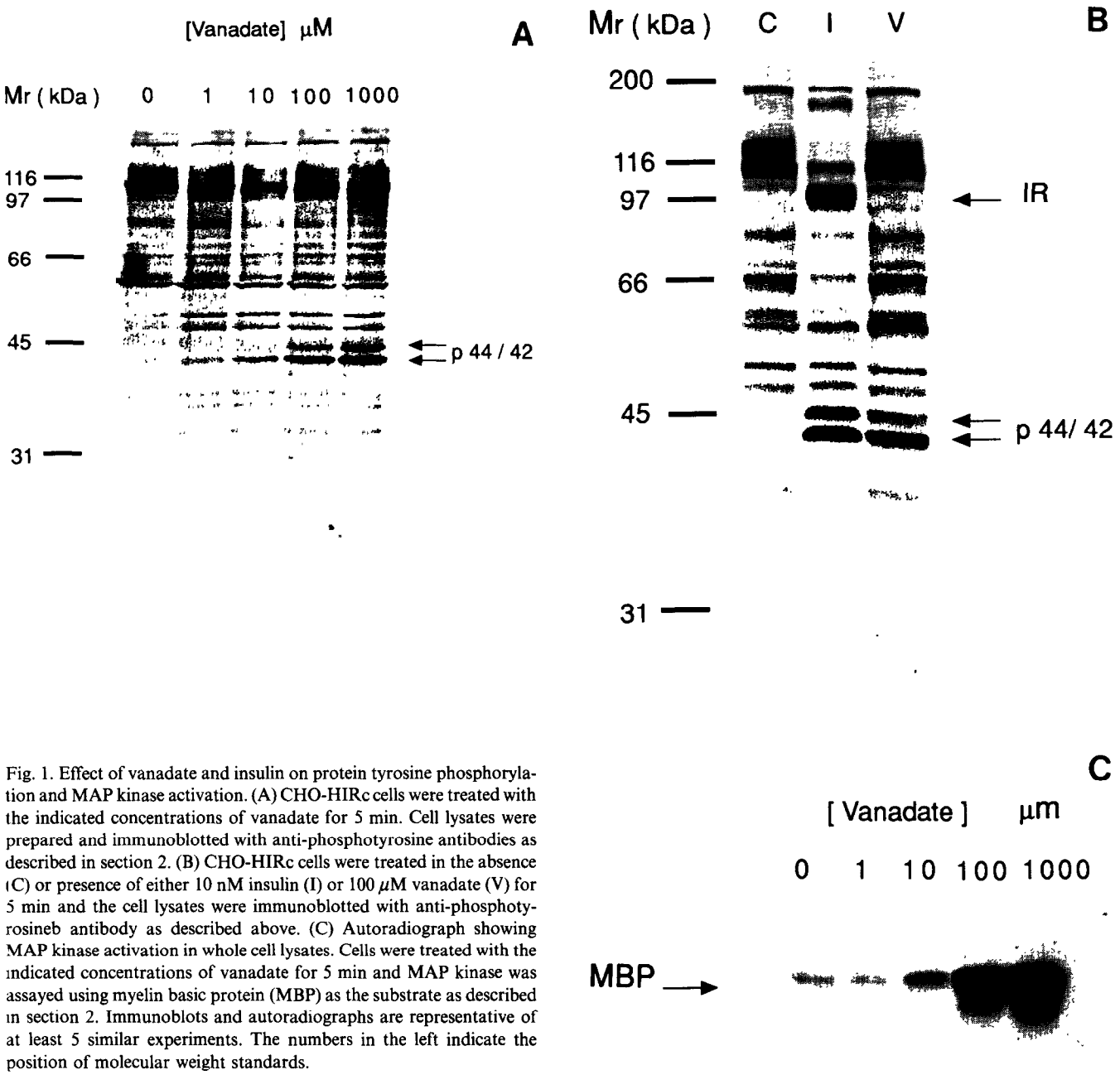

Fig. 1. Effect of vanadate and insulin on protein tyrosine phosphorylation and MAP kinase activation. (A) CHO-HIRc cells were treated with the indicated concentrations of vanadate for 5 min. Cell lysates were prepared and immunoblotted with anti-phosphotyrosine antibodies as described in section 2. (B) CHO-HIRc cells were treated in the absence (C) or presence of either 10 nM insulin (I) or 100 μM vanadate (V) for 5 min and the cell lysates were immunoblotted with anti-phosphotyrosineb antibody as described above. (C) Autoradiograph showing MAP kinase activation in whole cell lysates. Cells were treated with the indicated concentrations of vanadate for 5 min and MAP kinase was assayed using myelin basic protein (MBP) as the substrate as described in section 2. Immunoblots and autoradiographs are representative of at least 5 similar experiments. The numbers in the left indicate the position of molecular weight standards.

changed thereafter until 14 min (Fig. 2A). In contrast, however, the MAP kinase activation was maximal between 2 and 5 min of vanadate stimulation and declined at 14 min (Fig. 2B). It is interesting to note that despite the absence of a significant change in the degree of tyrosyl phosphorylation of p44 and p42 at 15 min (Fig. 2A), the extent of MAP kinase activation had significantly declined (Fig. 2B), probably due to dephosphorylation of threonine residues by protein serine/threonine phosphatases.

3.2. *Identification of p44/p42 as MAP kinases*

In order to ascertain whether p44 and p42 are indeed MAP kinases, the phosphotyrosyl proteins in lysates from insulin- and vanadate-treated cells were immunopurified on an anti-phosphotyrosine affinity column and the eluted phosphotyrosyl proteins were analyzed using a MAP kinase antibody. As shown in Fig. 3A, the antibody recognized two protein bands with apparent molecular sizes of 44 and 42 kDa corresponding to the two isozymic forms of $p44^{mapk}$ and $p42^{mapk}$, respectively [14]. These bands were detectable only in insulin- or vanadate-stimulated cells and not in unstimulated control cells (Fig. 3A), demonstrating that vanadate, similar to insulin, could increase the tyrosyl phosphorylation of $p44^{mapk}$ and $p42^{mapk}$. To examine whether there was a correlation between tyrosyl phosphorylation of $p44^{mapk}$ and $p42^{mapk}$ and MAP kinase activation, the im-

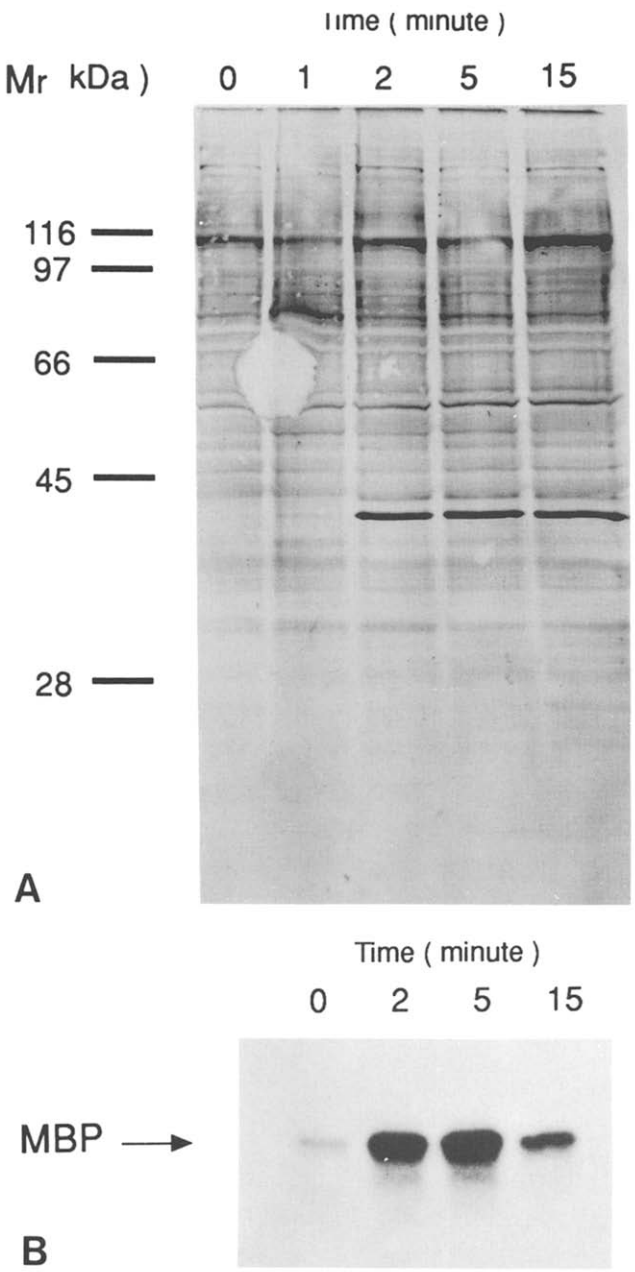

Fig. 2. Time-course of vanadate-stimulated protein tyrosine phosphorylation and MAP kinase activation in CHO-HIRc cells. (A) The cells were treated with 100 μM vanadate at the indicated time periods, and the cell lysates were prepared and immunoblotted with anti-phosphotyrosine antibody as described in section 2. (B) Autoradiograph showing MAP kinase activation. Cells were treated with 100 μM vanadate for indicated time periods and MAP kinase was assayed in whole cell lysates as in Fig. 1. Immunoblots and autoradiographs are representative of at least 5 similar experiments.

munoaffinity-purified phosphotyrosyl proteins obtained above were assayed for MAP kinase activity. As judged by the increased phosphorylation of MBP, insulin, as well as vanadate, stimulated the MAP kinase activity by 3 to 5-fold (Fig. 3B) as compared to controls. Since MAP kinase activation has been associated with a shift in its mobility on SDS-PAGE, we examined whether vanadate-mediated activation of MAP kinases resulted in a similar gel mobility shift. Indeed, as demonstrated by immunoblotting with MAP kinase antibodies, the lysates from vanadate- and insulin-treated cells exhibited a characteristic retardation in the mobility of $p44^{mapk}$ and $p42^{mapk}$, as compared to unstimulated control cells (Fig. 3C).

3.3. *Effect of vanadate on insulin receptor phosphorylation*

Since the immunoblotting experiments using anti-phosphotyrosine antibodies failed to detect tyrosyl phosphorylation of insulin receptor β-subunit in vanadate-stimulated HIRc cells, we further confirmed this effect by prelabeling the cells with [^{32}P]orthophosphate followed by stimulation with either vanadate or insulin. Immunoprecipitation of ^{32}P-labeled insulin receptor from these cells with a specific antibody to the β-subunit of the insulin receptor revealed that, although insulin treatment increased the tyrosyl phosphorylation of β-subunit of insulin receptor, a similar increase in tyrosyl phosphorylation was not detected in cell stimulated with 0.1 mM vanadate (Fig. 4).

To further verify the role of insulin receptor PTK in vanadate-mediated tyrosyl phosphorylation and activation of MAP kinases, CHO cells over-expressing a PTK-deficient insulin receptor (CHO-1018) were used. As shown in Fig. 5A, treatment of either parental CHO cells (CHO-NEO) or CHO-1018 with 100 μM vanadate for 5 min resulted in tyrosyl phosphorylation of two major proteins with molecular sizes of 44 and 42 kDa (p44/p42). Under these conditions, however, tyrosyl phosphorylation of insulin receptor β-subunit was not detected. These results are similar to those observed with CHO cells expressing wild-type insulin receptors (Fig 1A,1B). Furthermore, vanadate-stimulated tyrosyl phosphorylation of p44/p42 correlated with a corresponding activation of MAP kinase in CHO-NEO as well as in CHO-1018 cells. In both cell types vanadate stimulated the MAP kinase activity by about 4-fold, as compared to untreated control cells (Fig. 5B). In addition, analysis of cell lysates purified on an anti-phosphotyrosyl affinity column followed by immunoblotting with a MAP kinase antibody revealed that vanadate treatment of CHO-1018 and CHO-NEO cells increased the phosphotyrosyl content of the both $p42^{mapk}$ as well as $p44^{mapk}$ in a concentration-dependent manner (Fig. 5C).

4. Discussion

The results presented here demonstrate that the insulinomimetic agent, vanadate, stimulates the tyrosyl phosphorylation and activation of $p44^{mapk}$ and $p42^{mapk}$ in CHO cells over-expressing either a normal or PTK-deficient human insulin receptor. However, unlike insulin,

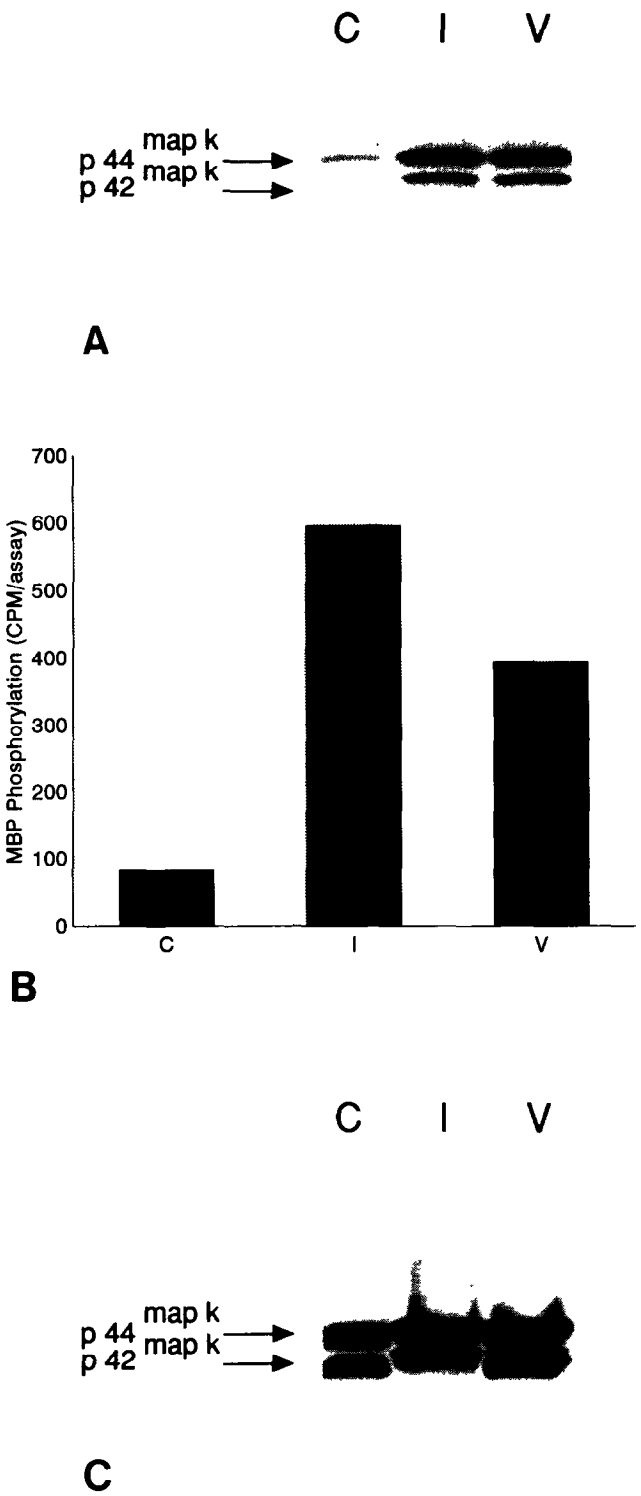

Fig. 3. Effect of insulin and vanadate on tyrosyl phosphorylation of MAP kinases. CHO-HIRc cells were incubated in the absence (C) or presence of either 10 nM insulin (I) or 100 μM vanadate (V) for 5 min and cell lysates were prepared. (A,B) Cell lysates were subjected to immunoaffinity purification on an anti-phosphotyrosine affinity column and the phosphotyrosine eluates were subjected to (A) immunoblotting using anti-MAP kinase antibody, and (B) MAP kinase assay using MBP as the substrate as described in section 2. (C) Gel mobility shift of $p44^{mapk}$ and $p42^{mapk}$. Lysates from cells treated with C, I or V were immunoblotted with MAP kinase antibody. These results are representative of 5 similar experiments.

the vanadate-mediated activation of $p44^{mapk}$ and $p42^{mapk}$ was not associated with the tyrosyl phosphorylation of insulin receptor β-subunit, even in CHO-HIRc. The lack of any effect of vanadate on insulin receptor tyrosyl phosphorylation is at variance with the results of Tamura et al. [10], who observed an increased tyrosyl phosphorylation of insulin receptor by vanadate. On the other hand, it is in agreement with several other studies [11–13,23]. In mouse diaphragm, in vivo administration of vanadate activated glycogen synthesis but failed to alter either the phosphorylation status or protein tyrosine kinase activity of the insulin receptor β-subunit [11]. Vanadate-mediated activation of MAP kinases was similar in all types of CHO cells used in this study and occurred even in the absence of the tyrosyl phosphorylation of insulin receptor β-subunit. It is interesting to note that CHO-1018 cells have been shown to be unresponsive in mediating the various metabolic or growth promoting effects of insulin [24,25]. It was also demonstrated recently that insulin fails to activate MAP kinases in CHO-1018 cells [26,27].

MAP kinase activation was also observed in CHO cells over-expressing PTK-deficient epidermal growth factor (EGF) receptors [28,29], and the authors indicated that MAP kinase was activated in response to a signal other than the EGF receptor, PTK. Our observations support this notion since MAP kinase activation oc-

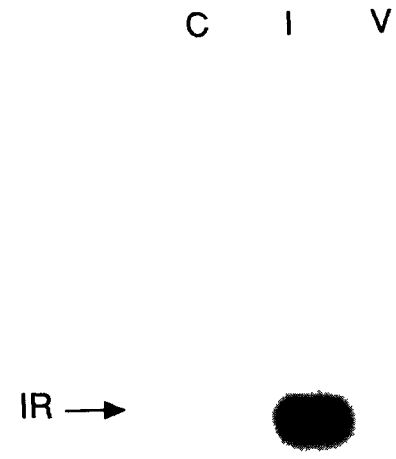

Fig. 4. Effect of vanadate on insulin receptor autophosphorylation. [^{32}P]Orthophosphate-pre-labeled CHO-HIRc cells were treated without (C) or with 100 nM insulin (I) or 100 μM vanadate (V) for 5 min. The cells were lysed and insulin receptor was immunoprecipitated with insulin receptor antibody as described in section 2. The immunoprecipitates were subjected to SDS-PAGE followed by KOH treatment and autoradiography, as described in section 2. The results are representative of at least 5 similar experiments.

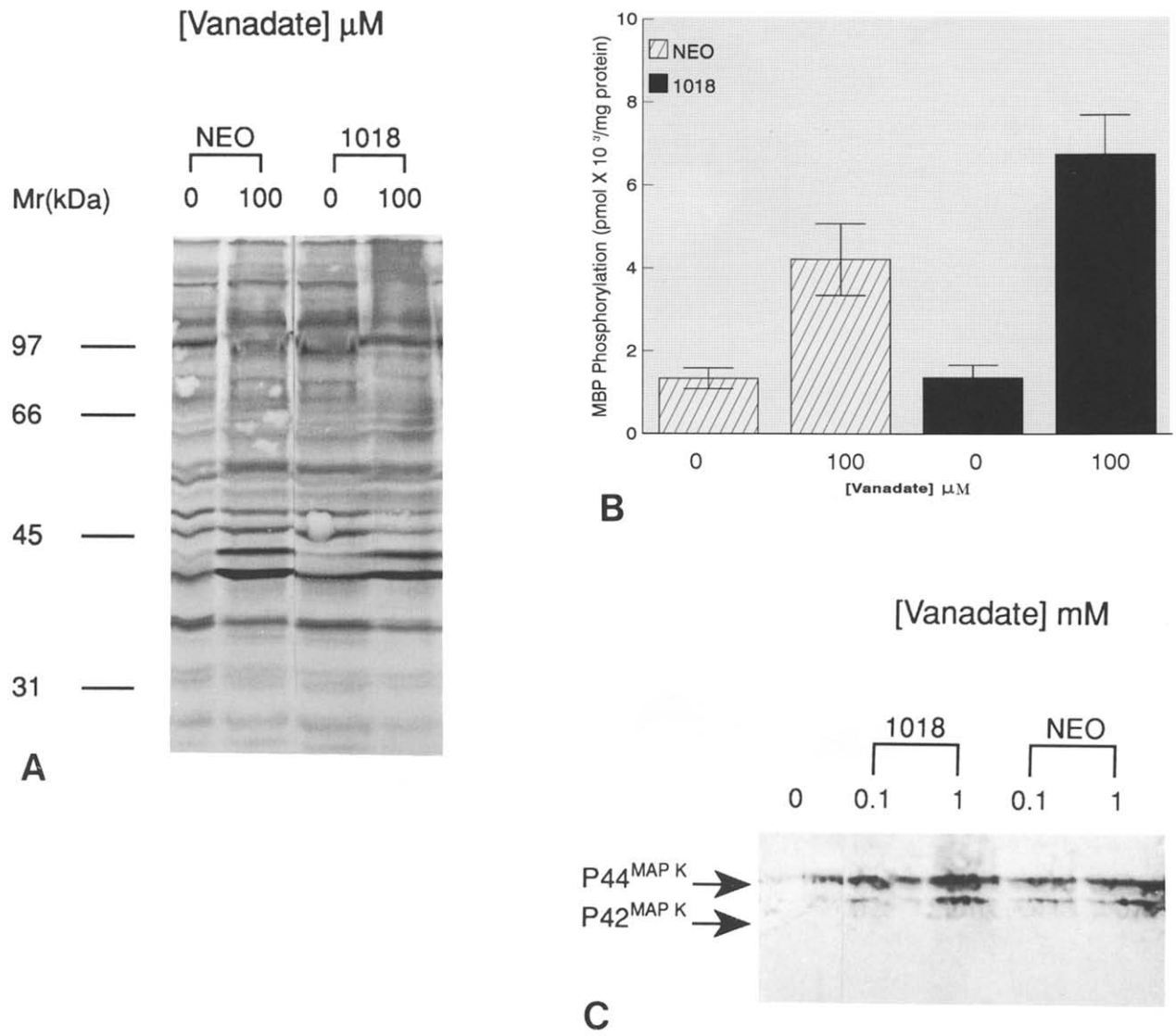

Fig. 5. Effect of vanadate on protein tyrosine phosphorylation and MAP kinase activation in parental (NEO) and insulin receptor protein tyrosine kinase-deficient CHO cells (CHO-1018). CHO-NEO and CHO-1018 cells were treated with 100 μM vanadate for 5 min. (A) Cell lysates were prepared and immunoblotted with anti-phosphotyrosine antibodies as described in section 2. (B) Cell lysates were subjected to MAP kinase assay using MBP as the substrate as described in section 2. Results represent the mean values ± S.E.M. of 4 (control) and 6 (vanadate-stimulated) independent experiments. (C) Cell lysates from A were subjected to immunoaffinity purification on an anti-phosphotyrosine antibody affinity column and the phosphotyrosine eluates were subjected to immunoblotting with MAP kinase antibody.

curred in the absence of receptor autophosphorylation. Our results on vanadate-mediated tyrosyl phosphorylation and activation of MAP kinases are intriguing and demonstrate for the first time vanadate per se could stimulate the tyrosyl phosphorylation of proteins. Earlier studies using several cell types have failed to detect any alteration in protein tyrosine phosphorylation with vanadate alone [30–34]. However, the reason for this discrepancy is not clear and may be due to the differences in the experimental conditions used. Recently, Scimeca et al. [35] demonstrated that vanadate was able to stimulate a kemptide kinase activity recovered in anti-phosphotyrosyl immunoprecipitates from NIH 3T3 cells over-expressing human insulin receptor. However, no attempt was made either to identify and characterise this kinase or to determine whether this stimulation was associated with tyrosyl phosphorylation of insulin receptor β-subunit [35].

The mechanism by which vanadate stimulates the tyrosyl phosphorylation and activation of MAP kinases is not known. Since vanadate is a potent inhibitor of protein tyrosine phosphatases (PTPases) [36], it is possible that, by preventing the dephosphorylation of tyrosyl phosphorylated insulin receptor β-subunit, it may cause an activation of insulin receptor PTK and mimic insulin in stimulating MAP kinases. However, the results presented here, as well as those of others [11–13], showing the lack of any effect of vanadate on the tyrosine phos-

phorylation of insulin receptor β-subunit, would argue against this possibility. It may still be possible that vanadate, by inhibiting a MAP kinase-specific PTPase(s), increases the tyrosyl phosphorylation as well as activation status of the MAP kinases. Recently, a MAP kinase-specific protein phosphatase which is inhibited by vanadate in vitro has been described [37]. However, it remains to be tested if this phosphatase is expressed in CHO cells and if the inhibition occurs in vivo.

Vanadate was shown to inhibit the Ca^{2+}/Mg^{2+}-ATPase and CA^{2+} transport system with an ID_{50} of 30 μM and 144 μM, respectively [38]. Moreover, it was demonstrated recently that mobilisation of intracellular sources of Ca^{2+} is sufficient to activate MAP kinases [39]. Therefore, another plausible explanation for our observations could be that by virtue of its ability to inhibit the Ca^{2+}/Mg^{2+}-ATPase pump vanadate might increase intracellular Ca^{2+} and thereby stimulate the MAP kinases activity.

Furthermore, upstream regulators of MAP kinases [15], such as MAP kinase kinase (MEK) or MEK kinase, may also be modulated by vanadate. Alternatively, it is also possible that vanadate diverges from the insulin signaling pathway and activates an unidentified kinase which triggers MAP kinase activation. One such candidate may be a novel cytosolic PTK which is stimulated solely be vanadate in rat adipocytes [40].

In summary, these results demonstrate that vanadate phosphorylates and stimulates the two isoforms of MAP kinases in the absence of insulin receptor β-subunit tyrosyl phosphorylation, and suggest that the PTK activity of the insulin receptor may not be required in signalling pathways leading to activation of MAP kinases by vanadate.

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