

# Pervanadate elicits proliferation and mediates activation of mitogen-activated protein (MAP) kinase in the nucleus

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**Abstract** There is growing evidence for the role of protein tyrosine phosphatases in controlling such fundamental cellular processes as growth and differentiation. Pervanadate is a potent inhibitor of protein tyrosine phosphatase which has been observed here to induce proliferation in C3H10T1/2 mouse fibroblasts. Pervanadate also translocated/activated p42/44 mitogen-activated protein (MAP) kinase to the cell nucleus. An almost similar pattern of nuclear p42/44 MAP kinase stimulation is seen with TPA. On the other hand, TPA treatment results in a rapid activation of cytosolic MAP kinase which declines with time. Thus pervanadate appears as a very useful tool for studying tyrosine phosphorylation.

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**Key words:** Protein tyrosine phosphatase; Protein tyrosine kinase; Pervanadate; Mitogen-activated protein kinase; TPA; Proliferation; Differentiation; C3H10T1/2 mouse fibroblast

## 1. Introduction

It is now well established [1,2] that some of the events occurring during proliferation are regulated by coordinate actions of protein tyrosine kinases and protein tyrosine phosphatases. One of the major signalling systems by which cells sense extracellular responses and transmit them intracellularly concerns the mitogen-activated protein kinase (MAPK) pathways [3–5]. Four subgroups of MAPKs have been identified: the ERKs (extracellular signal-regulated kinases) [6], Jun kinases [7], p38 MAP kinases [8], and BMK1/Erk-5 [9]. While ERKs are mainly activated by mitogenic signals, the JNK and p38 subclass of MAPKs are regulated as a sensor for stress signals [10]. The MAPKs are stimulated by dual phosphorylation [11] on threonine and tyrosine by upstream MAPK kinase (MAPKK). Thus one way by which growth stimuli transduce their growth promoting or proliferating signal is the activation of tyrosine kinases. On the other hand, inhibition [12–15] of tyrosine phosphatases has been shown to mimic some of the aspects of signal transduction that are normally triggered by tyrosine kinase activation pathway. Pervanadate has been reported to be a potent inhibitor of tyrosine phosphatase and thereby activates MAP kinase pathway [16]. In the study reported here we document that treatment of C3H10T1/2 mouse fibroblasts in culture with pervanadate leads to a proliferative response monitored by enhanced DNA synthesis and increased cell numbers. We also document that

pervanadate treatment of fibroblasts translocates/activates MAP kinase to the cell nucleus.

## 2. Materials and methods

### 2.1. Materials

C3H10T1/2 embryonic mouse fibroblasts were obtained from Dr. C.E. Wenner (Buffalo, NY). [ $\gamma$ - $^{32}$ P]ATP and 5-[ $^{125}$ I]iodo-2'-deoxyuridine were purchased from Amersham, antiphosphotyrosine antibody was from Transduction Laboratory, anti-ERK-2 antibody was from Santa Cruz Biotechnology, and all other reagents of highest purity grade were from Sigma France. Pervanadate (phosphotyrosine phosphatase inhibitor) was prepared as described by Zhao et al. [16].

### 2.2. Cell cultures

C3H10T1/2 embryonic mouse fibroblasts [17] were used at passage 15–22 at post-confluence quiescent state. Cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% inactivated fetal calf serum in a 5% CO<sub>2</sub> atmosphere at 37°C. The culture medium was renewed 5 days after plating on Petri dishes and cells attained confluence 9–10 days. Post-confluent cells were subjected to serum starvation for 48 h followed by treatment with TPA or pervanadate.

### 2.3. Preparation of cytosol and nuclear fractions

The cells were harvested in a medium containing 20 mM HEPES, 2 mM EDTA and 10% glycerol, sonicated 2 times (5 s each) with a 1 min interval between two sonications and centrifuged at 100 000×g. The resulting supernatant constituted the cytosolic fraction. Post-confluence cells were deprived of serum for indicated period. Cells were treated with TPA or pervanadate as required. Following treatments, cells were rinsed gently 3 times with a medium containing 20 mM Tris-HCl, pH 7.5, 1 mM MgCl<sub>2</sub>, and 1 mM dithiothreitol (medium A). Petri dishes, with attached cells, were frozen at –80°C for 2 h having a minimum amount of the above medium. After thawing on ice, cells were harvested in the medium A, centrifuged at 600×g for 10 min at 4°C and the resulting pellet was homogenized in 2 vol. of medium A supplemented with 1.3 M sucrose. Adequate amounts of medium A supplementing with 2.4 M sucrose were added to this suspension so as to give a final concentration of 2.2 M sucrose in the medium. This was homogenized with 4 up and down strokes in a Dounce homogenizer and centrifuged at 100 000×g for 20 min at 4°C in a Beckman TL 100 ultracentrifuge. The resulting pellet constituted isolated nuclei which were devoid of any plasma membrane contaminants as attested by marker enzymes activity [18]. The isolated nuclei were resuspended in a medium containing 20 mM Tris-HCl, pH 7.5, 4 mM MgCl<sub>2</sub>, 2 mM EDTA, 10% glycerol, 0.5% Triton X-100, 2 mM Na<sub>3</sub>VO<sub>4</sub>, 10 mM MgCl<sub>2</sub>, 100 μM ATP, 0.28 mg/ml myelin basic protein (MBP) and 3–5×10<sup>5</sup> cpm [ $\gamma$ - $^{32}$ P]ATP. Appropriate amounts of MAP kinase containing fraction (cytosolic/nuclear) was incubated for 15 min at 30°C. After incubation, proteins were precipitated with 20% TCA and radioactivity was determined by Cerenkov spectrometry.

### 2.4. MAP kinase activity

The standard assay medium contained: 20 mM HEPES, pH 7.5, 20 mM β-glycerophosphate, pH 7.5, 20 mM NaF, 2 mM EDTA, 2 mM Na<sub>3</sub>VO<sub>4</sub>, 10 mM MgCl<sub>2</sub>, 100 μM ATP, 0.28 mg/ml myelin basic protein (MBP) and 3–5×10<sup>5</sup> cpm [ $\gamma$ - $^{32}$ P]ATP. Appropriate amounts of MAP kinase containing fraction (cytosolic/nuclear) was incubated for 15 min at 30°C. After incubation, proteins were precipitated with 20% TCA and radioactivity was determined by Cerenkov spectrometry.

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A study in C3H10T1/2 mouse fibroblasts in culture.

### 2.4.1. Western blotting

Fractions endowed with MAP kinase activity were mixed with electrophoresis sample buffer and boiled for 5 min at 90°C. Proteins were separated on SDS-PAGE (10% polyacrylamide). After electrophoresis proteins were transferred to nitrocellulose membranes using BioRad semi-dry apparatus. The immunoblot was revealed using an anti-ERK-2 antibody (1:1000 dilution), or antiphosphotyrosine antibody (1:250 dilution) as primary antibody employing alkaline phosphatase-conjugated secondary antibody. The immunoblot was developed using NBT-BCIP reagent.

### 2.5. DNA synthesis

For  $^{125}\text{I}$ -labelled uridine incorporation assay, cells were plated in 24-well plates (Falcon, Oxnard, CA) at a density of  $1.6 \times 10^6$  cells per well. Cells were deprived of serum for 48 h followed by various treatments. For instance cells were treated with 10% fetal calf serum, 160 nM TPA (without serum for 10 min) 12.5  $\mu\text{M}$  freshly prepared pervanadate (with 10% serum for 2 h). Cells were pulsed labelled with  $1.2 \times 10^5$  cpm of iodinated uridine for 2 h. Cells were centrifuged at  $600 \times g$  for 10 min and radioactivity was determined by spectrometry counting.

### 2.5.1. Cell counting

Cells were plated in 30 mm Petri dishes at a density of  $1.25 \times 10^5$  cells per dish and allowed to grow. Cells were serum-deprived for 48 h, followed by effector treatment as mentioned above. Cells were rinsed extensively and counted after Trypan Blue staining.

## 3. Results

### 3.1. Pervanadate-induced proliferation

Fig. 1A illustrates enhanced DNA synthesis as monitored by  $^{125}\text{I}$ iodo-2'-deoxyuridine incorporation upon effector treatment. Pervanadate caused maximum uridine incorporation as compared with TPA treatment or treatment with fetal calf serum. The uridine incorporation was dependent upon the time employed to continue cell growth after an initial short-term effector treatment, with maximum uridine incorporation occurring at 15 h.

Both pervanadate or TPA treatment of fibroblasts resulted

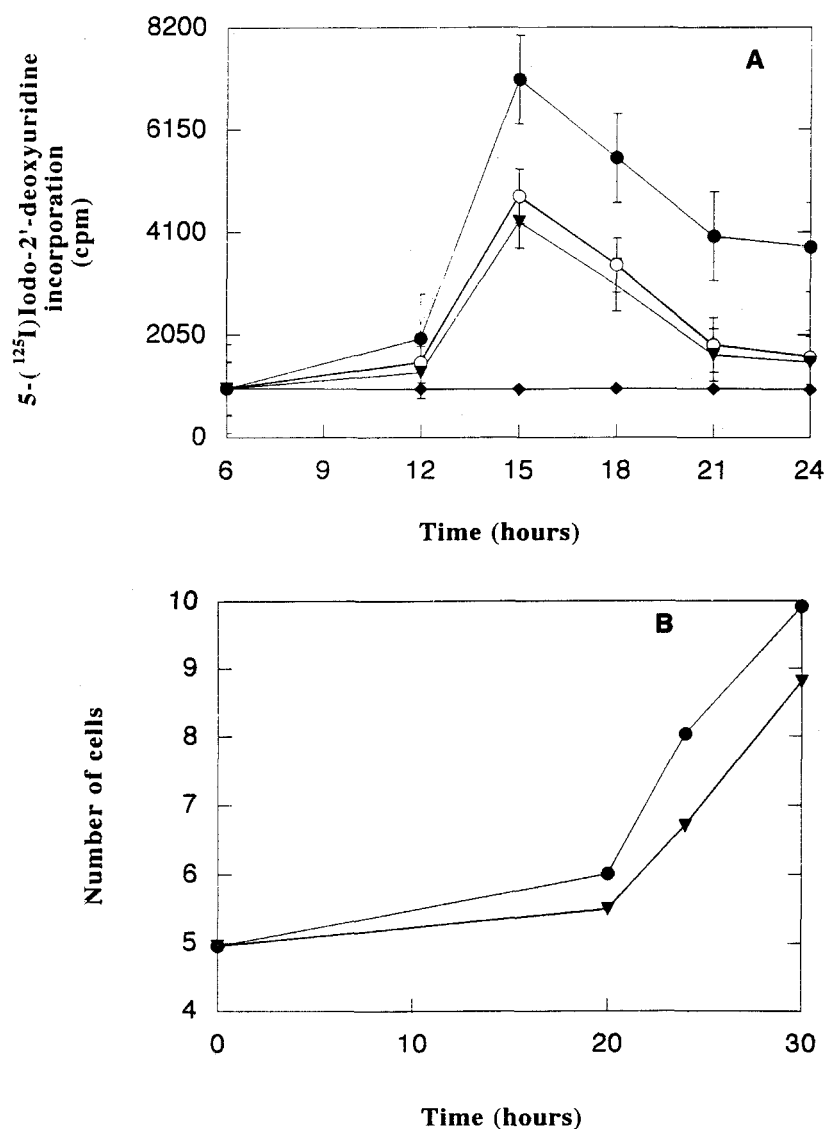


Fig. 1. Pervanadate-induced proliferation. Mouse fibroblasts were treated with pervanadate (●), serum (○), phorbol ester (▲) as described under Section 2. Enhanced DNA synthesis was determined by  $^{125}\text{I}$ -labelled deoxyuridine incorporation (A) and cells were counted after Trypan Blue staining (B). Concentrations of effectors were: pervanadate, 12.5  $\mu\text{M}$ ; phorbol ester, 160 nM; serum 10%.

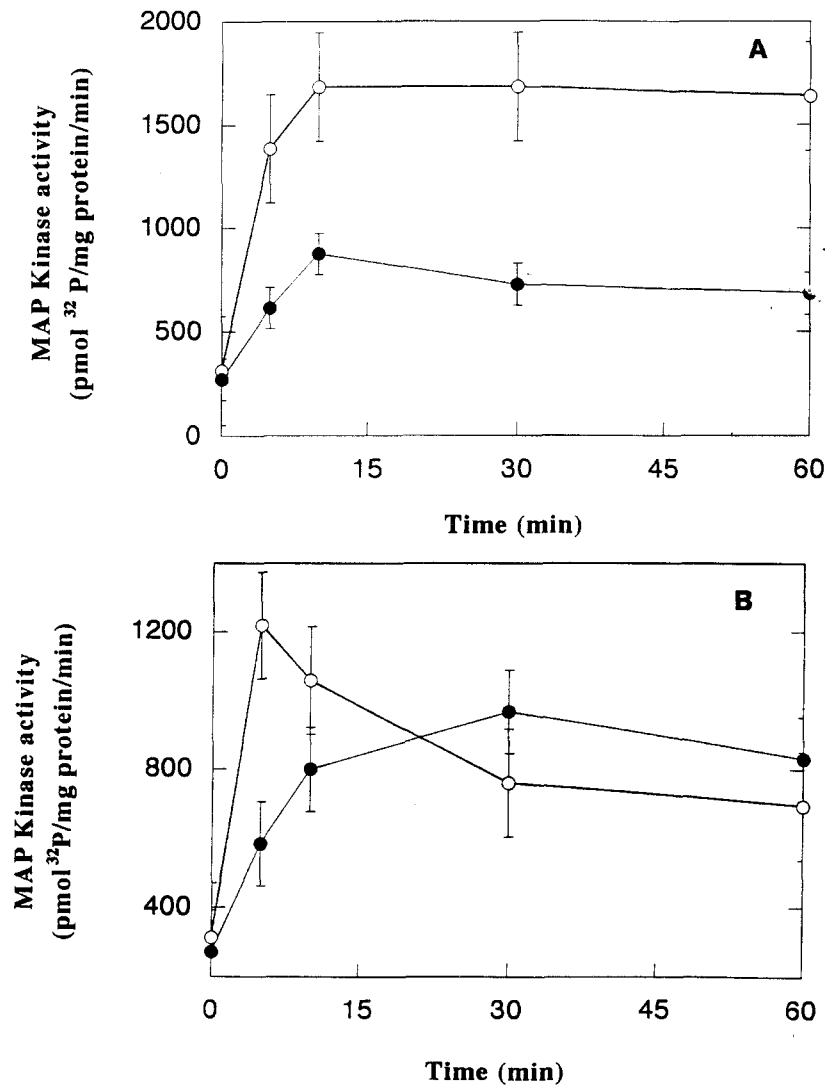


Fig. 2. p42/44 MAP kinase activity. MAP kinase activity was determined in the cytosolic (○) and nuclear fractions (●) derived from pervanadate (A) or TPA (B) treated cells as described under Section 2. Standard assay medium contained in (mM): 20 HEPES, pH 7.5; 20 β-glycerophosphate, pH 7.5; 20 NaF; 2 EDTA; 2 Na<sub>3</sub>VO<sub>4</sub>; 10 MgCl<sub>2</sub>; 100 μM ATP, 3–5 × 10<sup>5</sup> cpm [γ-<sup>32</sup>P]ATP and 0.28 mg/ml MBP. The incubation was carried out at 30°C for 15 min.

in increased cell numbers (Fig. 1B) which further confirmed the proliferative response of these effectors in C3H10T1/2 fibroblasts.

### 3.2. Pervanadate stimulates and translocates MAP kinase to the nucleus

C3H10T1/2 mouse fibroblasts were treated with pervanadate (Fig. 2A) and TPA (Fig. 2B) and MAP kinase activity was determined in the cell cytosol and the cell nucleus. Pervanadate treatment resulted in sustained activation of MAP kinase in the cytosolic and nuclear fractions. The activities reached plateau level within 10 min of cell treatment and stayed thereafter. The TPA treatment stimulated MAP kinase activity in the cytosolic fraction within 5 min, which declined from then on, attaining to a lower level in 30 min. The nuclear MAP kinase activity showed a biphasic response (Fig. 2B). There was a tendency for a transient stimulation which appeared to follow a slow rise in activity attaining maximum stimulation in 30 min. A similar pattern of nuclear MAP

kinase stimulation was seen either with pervanadate or TPA treatment of cells.

Activation of MAP kinase was also supported by Western blot data obtained employing anti-ERK-2 antibody as well as antiphosphotyrosine antibody. A time-dependent activation of MAP kinase was seen upon pervanadate treatment in the cytosol (Fig. 3A,B) and in the nucleus (Fig. 3C,D). Slow mobility of ERK-2 on electrophoresis due to stimulation of MAP kinase within the time frame of enhanced activity found full confirmation from the antiphosphotyrosine immunoblotting (Fig. 3B,D) results.

## 4. Discussion

Activation of protein tyrosine kinases is a critical component of signalling pathways that control cell proliferation and differentiation. The importance of protein tyrosine phosphatase in the regulation of cell function has recently been emphasized [1,19,20]. Most interesting results were advanced by

Fischer demonstrating that inactivation of protein tyrosine phosphatases by pervanadate produced drastic tyrosine phosphorylation of cellular proteins, which ultimately turn on the MAP kinase pathway [16]. Furthermore, Cohen [20] has come up with the still striking observation when he and his colleagues observed tyrosine phosphorylation of a number of proteins, as well as nuclear translocation of a number of tyrosine phosphorylated stat (signal transducer and activator of transcription) proteins, upon intraperitoneal injection of pervanadate into mice. Thus, Cohen [20] has established that the control of protein tyrosine phosphatase activity may be of greater significance in cell signaling than heretofore realized. However, the role of pervanadate in inhibiting protein tyrosine phosphatase with respect to cell proliferation has not been addressed adequately.

The present study demonstrates that pervanadate can serve as an inducer of cell proliferation in C3H10T1/2 mouse fibroblasts in culture. The effect of pervanadate on cell proliferation was comparable with that of TPA or serum. DNA synthesis was enhanced (Fig. 1A), and cell numbers were increased (Fig. 1B). Therefore, inactivation of protein tyrosine phosphatase leads to a proliferative response in this cell system. These results establish for the first time that pervanadate is a potent inducer of mouse fibroblasts proliferation. They confirm that a balance between protein tyrosine phosphatases and tyrosine kinase activity [21,22] is essential for the regulation of cell proliferation.

Another important message that is conveyed by the data reported in this article is that treatment of mouse fibroblasts in culture by pervanadate leads to nuclear activation and

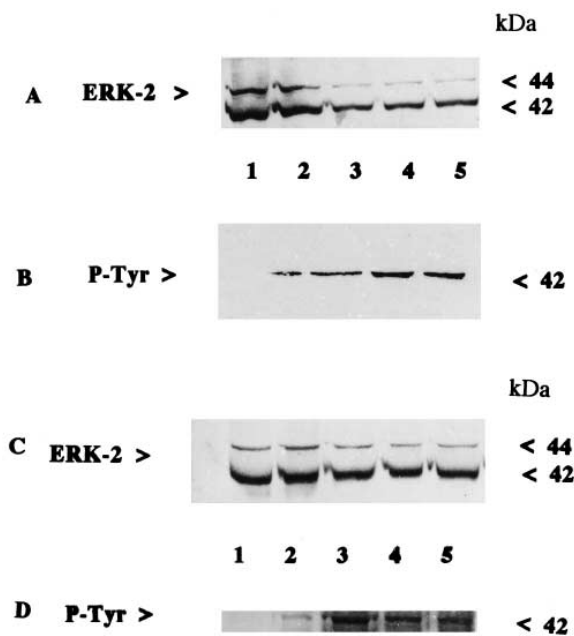


Fig. 3. Western blotting after pervanadate treatment. Cytosolic (A,B) or nuclear (C,D) fractions were separated on SDS-PAGE and transferred to nitrocellulose membranes. The nitrocellulose membranes were subjected to Western blotting employing anti-ERK antibodies (A,C) or anti-phosphotyrosine antibodies (B,D). Lane 1: control; lanes 2-5 pervanadate treatment for 5, 10, 30 and 60 min, respectively. Antiphosphotyrosine antibody recognized the ERK-2 only when the enzyme was activated. For Western blotting in A and C, 8  $\mu$ g protein was loaded in each lane; in B and D, 16  $\mu$ g protein was loaded.

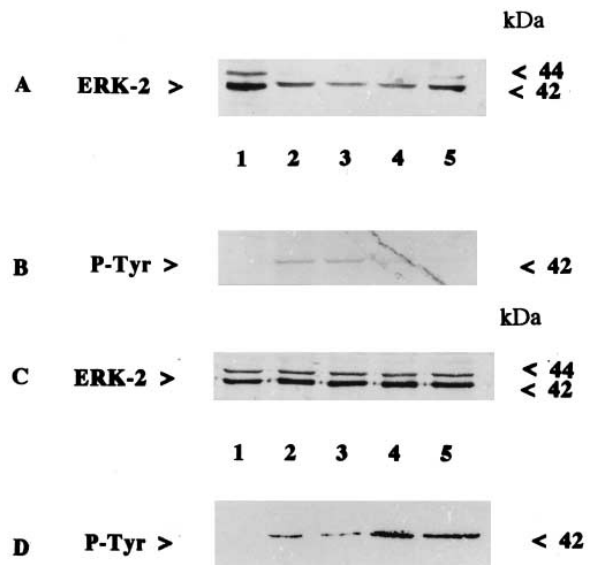


Fig. 4. Western blotting after TPA treatment. A,C: Western blots obtained using anti-ERK antibodies. B,D: Followed by anti-phosphotyrosine antibodies. Experimental conditions, amount of protein loaded to each lane and time of treatment corresponding to each lane were the same as described for Fig. 3.

translocation of p42/44 MAP kinase (Fig. 2A,B). A similar profile was observed for TPA stimulation of nuclear MAP kinase activity. However, the pattern of cytosolic p42/44 kinase activation by pervanadate is at variance with that induced by TPA stimulation. Pervanadate induced MAP kinase activation was sustained up to 60 min both in the cytosol and in the nuclear fraction. On the other hand, in the cytosol MAP kinase activation by TPA was maximum 5 min after treatment and declined progressively thereafter.

These results are compatible with the stimulation/translocation of MAP kinase to the nucleus [23,24] both by TPA and pervanadate. However, in the case of TPA stimulation, the phosphatases [25,26] which physiologically inactivate MAP kinase in the cytosol are not inhibited. Thus, as observed here, the activity of MAP kinase in the cytosol would be expected to decline. Several dual specificity phosphatases which inactivate MAP kinase have been identified and so far they have not been found in the nucleus. It may be safely argued here that in the presence of pervanadate, as these phosphatases are inhibited, the cytosolic activity of MAP kinase ought to be sustained. This is precisely what we have observed.

The activation of p42/44 MAP kinase was fully supported by and immunoreactivity with antiphosphotyrosine antibody, and the gel mobility shift (Figs. 3 and 4). The antiphosphotyrosine antibody which recognized only the activated p42/44 MAP kinase, confirmed that a time-dependent activation of MAP kinase occurred upon pervanadate treatment of mouse fibroblasts in culture. The time course of MAP kinase phosphorylation on tyrosine residues was the same as that of MAP kinase activity. This parallelism holds for both pervanadate and TPA stimulation, except that the intensity of immunoreactivity which was much more pronounced after pervanadate treatment of cells. These results are further evidence [16] showing that pervanadate is a potent activator of MAP kinase through inhibition of protein tyrosine phosphatase.

In conclusion, pervanadate is able to induce cell proliferation and activate MAP kinase in the nucleus. These results

further strengthen the role of protein tyrosine phosphatase in nuclear signalling and cell proliferation.

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