Pervanadate inhibits mitogen-activated protein kinase kinase-1 in a p38^{MAPK}-dependent manner

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Received 10 March 1998; revised version received 8 April 1998

Abstract In baboon smooth muscle cells (SMCs), pervanadate has a biphasic dose-dependent effect on MEK-1 activity. After a 30 min incubation period, low concentrations (1–10 μM) activate, while higher doses (30–100 μM) fail to stimulate MEK-1. One possibility is that higher doses of pervanadate induce an additional signaling pathway that inhibits MEK-1. Three lines of investigations provide support for the conclusion that this inhibitory effect is mediated by p38^{MAPK}. First, pervanadate induces p38^{MAPK} activity at concentrations that fail to activate MEK-1. Second, pervanadate-stimulated p38^{MAPK} activity is maximal after a 10 min incubation, at a time, when MEK-1 activity disappears. Third, addition of the specific p38^{MAPK} inhibitor SB203580 preserves MEK-1 activation by 100 μM pervanadate. The inhibitory effect of p38^{MAPK} is probably not due to a phosphorylation of MEK-1 although we can not rule out that other p38^{MAPK} isoforms such as SAPK3 and SAPK4 may be involved, and may directly phosphorylate and inhibit MEK-1.

Key words: Signal transduction; Smooth muscle; Oxidative stress; SB203580

1. Introduction

Time-dependent activation of mitogen-activated protein kinases (MAPKs) is integral to many signal transduction pathways (reviewed in [1]). Members of this subfamily of protein kinases include the extracellular signal-regulated kinases (ERK-1 and -2) which are involved in proliferation and differentiation, and the stress-activated protein kinases (SAPKs) which mediate cellular stress signaling. SAPKs consist of at least two subfamilies, the jun kinases and p38^{MAPK} (reviewed in [1,2]). MAPKs are activated by specific upstream kinases (MAPK kinases or MEKs) which phosphorylate both threonine and tyrosine residues in a conserved TXY motif in kinase subdomain VIII (reviewed in [3]). Growth factor-induced activation of ERK-1 and -2 is a crucial event for both cell cycle progression and survival. In contrast, SAPKs are activated by stress factors, such as reactive oxygen species or UV light, which inhibit proliferation. In some cell types, SAPKs also play an active role in apoptotic pathways [4–7]. Although direct cross talk between different MAPK pathways has been suggested [8,9] a direct interaction has not yet been identified. This study demonstrates that pervanadate-induced p38^{MAPK} activity has an inhibitory effect on the ERK-1, -2 activator MEK-1 in aortic baboon smooth muscle cells.

2. Materials and methods

2.1. Materials

Antibodies against MEK-1 were purchased from Transduction Laboratories (Lexington, KY). Antibodies against p38^{MAPK} and Raf-1 were from Santa Cruz Biotechnology (Santa Cruz, CA). MAPKAPK-2 substrate peptide and antibodies were from Upstate Biotechnology (Lake Placid, NY). Protein A agarose was from Sigma (St. Louis, MO), tissue culture media and fetal calf serum were from GIBCO-BRL (Gaithersburg, MD). SB203580 was from Calbiochem (La Jolla, CA) and ^{32}P-γ-ATP from Du Pont-New England Nuclear (Boston, MA). Catalytically inactive, histidine-tagged mutants of ERK-2 (K52R), and MEK-1 (K97M) were prepared with minor modifications as described [10,11]. Purified GST-ATF-2 was a kind gift from Jonathan Graves (Department of Pharmacology, University of Washington, Seattle, WA). A stock solution of 50 mM pervanadate was freshly prepared by mixing equal volumes of 0.1 M Na-orthovanadate and 0.1 M hydrogen peroxide.

Buffer HEB: 25 mM HEPES-NaOH, pH 7.5, 10% glycerol, 5 mM EDTA, 5 mM EGTA, 150 mM NaCl, 100 mM Na-pyrophosphate, 50 mM NaF, 1 mM Na-vanadate, 1 mM benzamidine, 0.1% 2-mercaptoethanol, 1% Triton X-100, 1 μM pepstatin A, 2 μg/ml leupeptin and 20 kallikrein inhibitor units/ml aprotinin; kinase buffer: 20 mM HEPES-NaOH, pH 7.5, 20 mM $MgCl_2$, 0.1% 2-mercaptoethanol; TTBS buffer: 25 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.1% Triton X-100, 0.1% 2-mercaptoethanol.

2.2. Cell culture

Baboon aortic smooth muscle cells were prepared as described previously [12]. Cells in passage numbers ranging between 5 and 25 were grown in Dulbecco's modified Eagle's medium supplemented with 10% FCS, 200 U/ml penicillin and 0.2 mg/ml streptomycin.

2.3. Kinase assays

Subconfluent (~70%) SMCs were starved for 2–3 days in serum-free media before stimulation as indicated in the text. Cells were harvested and extracted in buffer HEB as described [13]. One μg of antibody and 20 μl of protein A sepharose slurry (1 mg/ml protein A) were added to 0.25–0.5 mg extracted protein, depending on the experiment. The samples were stirred overnight at 4°C. The beads were washed once in buffer HEB followed by TTBS and kinase buffer. The kinase reaction was performed on the beads in kinase buffer containing 1 μg of protein substrate (or 0.2 mM substrate peptide in MAPKAPK-2 assays), and 0.1 mM ATP (5000 cpm/pmol). The assay mix was incubated for 30–45 min at room temperature. For MEK-1, p38^{MAPK}, and Raf-1 assays, the reactions were terminated by adding 10 μl 4× Laemmli buffer and the samples were subjected to SDS-PAGE [14]. The gels were stained with Coomassie-blue to monitor equal loading, dried, and the extent of substrate phosphorylation

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Abbreviations: ERK, extracellular signal-regulated kinase; FCS, fetal calf serum; MAPK, mitogen-activated protein kinase; MAPKAPK, MAPK-activated protein kinase; MEK, MAPK/ERK kinase; PDGF, platelet-derived growth factor; ROS, reactive oxygen species; SAPK, stress-activated protein kinase; S.D., standard deviation; SMC, smooth muscle cell

PII S0014-5793(98)00448-7

was determined by phosphorimaging (facility at the University of Washington, Seattle, WA) or densitometric analysis of the autoradiograph. Assays were performed in duplicates. The MAPKAPK-2 samples were spun and the supernatant spotted on P81 paper (Whatman Inc., Clifton, NJ). Following five washes with 0.75% phosphoric acid, the paper-bound radioactivity was determined by scintillation counting.

3. Results and discussion

3.1. Pervanadate has a biphasic effect on MEK activity

Pervanadate, a mixture of vanadate and hydrogen peroxide, is widely used as potent phosphatase inhibitor [15,16] and has recently been shown to strongly activate the ERK/MEK signaling module in HeLa cells [17]. Surprisingly, a biphasic dose-dependent effect of pervanadate on MEK-1 activation was observed in smooth muscle cells (SMCs) following a

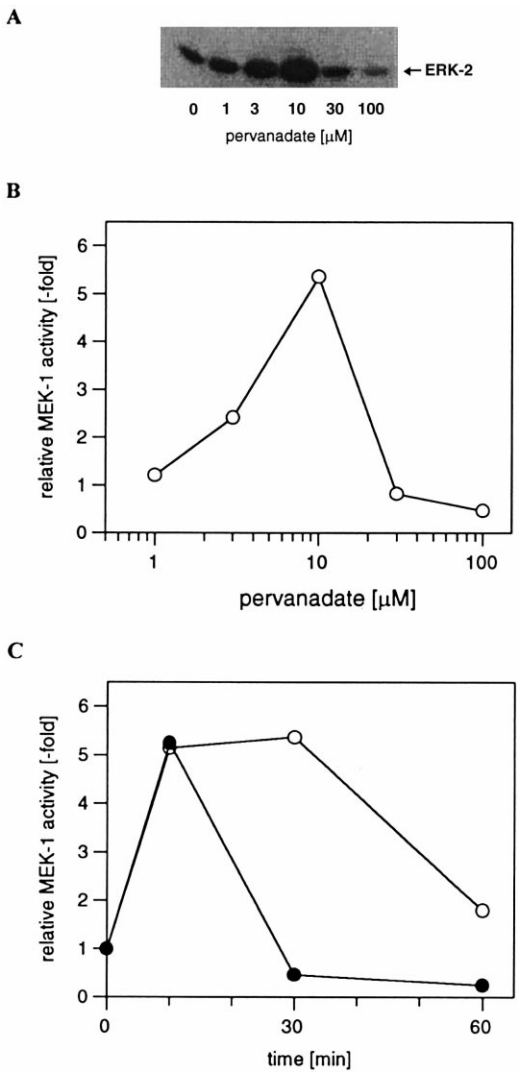

Fig. 1. Effects of pervanadate on MEK-1. MEK-1 activities were determined with recombinant ERK-2 as substrate in extracts from SMCs that have been stimulated with pervanadate. A, B: SMCs were incubated for 30 min with 1, 3, 10, 30, and 100 μM pervanadate, respectively. A: Autoradiograph of a representative experiment. B: Graph representing mean values of 2–3 independent experiments. C: SMCs were incubated with 10 μM (open circles) and 100 μM (solid circles) pervanadate for 10, 30, and 60 min respectively. Mean values of 2–3 independent experiments are shown.

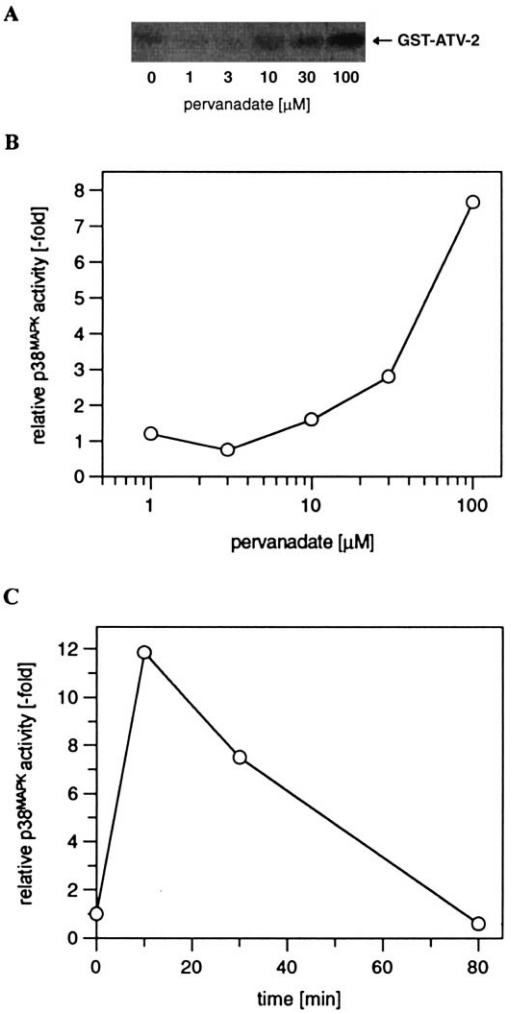

Fig. 2. Effects of pervanadate on $p38^{MAPK}$. $p38^{MAPK}$ activities were determined with GST-ATF-2 as substrate in extracts from SMCs that have been stimulated with pervanadate. A, B: SMCs were incubated for 10 min with 1, 3, 10, 30, and 100 μM pervanadate, respectively. A: Autoradiograph of a representative experiment. B: Graph representing mean values of 2–3 independent experiments. C: SMCs were incubated with 100 μM pervanadate for 10, 30, and 80 min respectively. Mean values of two independent experiments are shown.

30 min incubation period. Concentrations of pervanadate up to 10 μM activated MEK-1, whereas concentrations greater than 10 μM did not (Fig. 1A, B). Time course experiments with 10 and 100 μM pervanadate revealed that 100 μM pervanadate was capable of activating MEK-1 at 10 min, but kinase activity was short-lived and completely disappeared after 30 min (Fig. 1C). In contrast, MEK-1 activity induced by 10 μM pervanadate peaked at 30 min and remained above basal levels after 1 h (Fig. 1C). One explanation for these kinetic differences may lie in the possibility that pervanadate in concentrations higher than 10 μM initiates an additional signaling pathway that can inhibit MEK-1. It is noteworthy that vanadate, but not peroxide, activated MEK-1 to a similar extent when compared to pervanadate. Vanadate, however, completely lacked any inhibitory effect at higher concentrations. These findings suggest that the effects of pervanadate on MEK-1 may require both the activatory action of vanadate and an inhibitory action of peroxide.

3.2. MEK-1 inhibition by pervanadate is mediated by p38^{MAPK}

As p38^{MAPK} is activated by ROS [18,19], we tested whether this kinase is also stimulated by pervanadate in SMCs, and thus, may play a role in inhibiting MEK-1. Pervanadate activated p38^{MAPK} in a dose-dependent fashion with kinase activity first detectable at 10 μM pervanadate. Higher doses of pervanadate, 30 and 100 μM, further stimulated p38^{MAPK} up to 8-fold above basal levels (Fig. 2A, B). p38^{MAPK} activity reaches its maximum 10 min after the addition of pervanadate (Fig. 2C); at that time, the loss of MEK-1 activity is observed (Fig. 1C). Thus, the dose response and the kinetics of pervanadate-induced p38^{MAPK} agree with the possibility that p38^{MAPK} blocks MEK-1 activation.

To examine the hypothesis that p38^{MAPK} may block MEK-1, we employed the specific p38^{MAPK} inhibitor SB203580 [20,21]. SMCs were preincubated with various concentrations of SB203580 and stimulated for 30 min with 100 μM pervanadate. Besides MEK-1, we also determined the activity of MAPKAPK-2, which is a downstream substrate for p38^{MAPK}, to monitor the efficacy of the p38^{MAPK} inhibitor. Under these conditions, SB203580 (0.8–25 μM) caused MEK-1 activation in a dose-dependent manner with a 6-fold stimulation in the presence of 25 μM inhibitor (Fig. 3). This suggested to us that the suppression of p38^{MAPK} was sufficient to allow MEK-1 activation by 100 μM pervanadate. As expected, in the absence of SB203580, pervanadate stimulated MAPKAPK-2 activities. This stimulation was abolished by SB203580 concentrations (0.8–2.5 μM) lower than those required to induce MEK-1 activation (Fig. 3). This difference may be explained by a role of p38^{MAPK} isoforms, such as SAPK3 and SAPK4 [2,22], which are less sensitive to SB203580 and may also contribute to the inhibitory effect on MEK-1. However, we can not rule out that in addition to blocking p38^{MAPK}, SB203580 might have non-specific effects at higher doses. In control experiments, where 25 μM SB203580 were added to 10 μM pervanadate, the p38^{MAPK} inhibitor had no activatory effect on MEK-1 (data not shown).

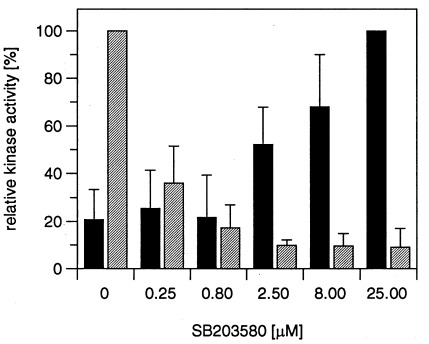

Fig. 3. Effect of the p38^{MAPK} inhibitor SB203580 on MEK-1 and MAPKAPK-2 activities in the presence of 100 μM pervanadate. SMCs were preincubated for 1 h with 0.25% DMSO and various concentrations of SB203580 as indicated. Cells were stimulated for 30 min with 100 μM pervanadate and extracted. MEK-1 activities were determined with recombinant ERK-2 as substrate and MAPKAP kinase-2 was measured as described in Section 2.3. Data (mean ± S.D. of independent experiments) are presented as percent activity where the highest activities obtained are 100%. Solid bars: MEK-1 ($n = 3$); hatched bars: MAPKAPK-2 ($n = 2$).

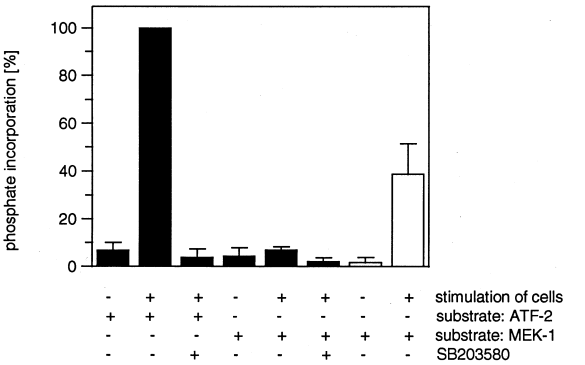

Fig. 4. p38^{MAPK} does not significantly phosphorylate MEK-1. p38^{MAPK} and Raf-1 were immunoprecipitated from starved SMCs and cells that were stimulated for 10 min with 100 μM pervanadate and 10 ng/ml PDGF-BB, respectively. p38^{MAPK} activities (black bars) were measured with GST-ATF-2 and MEK-1 as substrates in the absence and presence of 8 μM SB203580. As control, MEK-1 was used as substrate for Raf-1 (white bars). Data are presented as percent phosphate incorporated, with GST-ATF-2 being 100% (mean ± S.D., $n = 3$ except $n = 2$ for Raf assays and assays including SB203580).

3.3. p38^{MAPK} does not significantly phosphorylate MEK-1

p38^{MAPK} was immunoprecipitated from pervanadate-stimulated cells and kinase activities were determined towards ATF-2 and MEK-1 in the absence and presence of SB203580. As positive control for the MEK-1 preparation, we assayed Raf-1 following immunoprecipitation from PDGF-BB-treated SMCs. Stimulation of p38^{MAPK} resulted in a 15-fold increase in ATF-2 phosphorylation but only in a 1.5-fold increase in MEK-1 phosphorylation. Under identical assay conditions, stimulation of Raf-1 yielded a 20-fold increase in MEK-1 phosphorylation (Fig. 4). As expected, in the presence of SB203580, p38^{MAPK} activity was abolished. We estimated that p38^{MAPK} incorporated less than 0.1 mol phosphate/mol into MEK-1. Furthermore, immunoprecipitated, PDGF-BB-activated MEK-1 was not inhibited by preincubation with pervanadate-stimulated p38^{MAPK} (data not shown). From these data, we conclude that p38^{MAPK} does not exert its inhibitory effect on MEK-1 by directly phosphorylating the kinase. As mentioned above, it is possible, however, that other p38^{MAPK} isoforms such as SAPK3 and SAPK4 are involved and may phosphorylate and inhibit MEK-1.

3.4. Summary

We show that low concentrations of pervanadate result in a sustained activation of MEK-1, whereas high concentrations only transiently activate MEK-1. This difference can be explained by an inhibitory effect of pervanadate that is, at least partially, mediated by a member of the p38^{MAPK} family. This inhibitory effect on MEK-1 might be cell type specific since it has not been observed in HeLa cells [17]. In those studies, however, MEK-2 was investigated rather than MEK-1. It is therefore possible that only the MEK-1 isoform is sensitive to pervanadate inhibition. In SMCs, we could not test this possibility, as MEK-2 activity is not detectable following pervanadate stimulation (not shown). It is an intriguing possibility that the blockade of MEK-1 activation by p38^{MAPK} is part of the mechanisms by which DNA damaging agents cause growth inhibition. Clearly, future work has to be directed towards the elucidation of the molecular basis for p38^{MAPK}-

mediated MEK-1 inhibition, as well as the identification of the $p38^{MAPK}$-dependent signaling element that is executing the inhibitory effect.

Acknowledgements: We thank Melanie Cobb (University of Texas, Dallas, TX) and Nathalie Ahn (University of Colorado, Boulder, CO) for the constructs encoding the histidine-tagged ERK-2 and MEK-1 mutants, Jonathan Graves (University of Washington, Seattle, WA) for purified GST-ATF-2 protein, Zymogenetics (Seattle, WA) for PDGF-BB, and Mike Mikiska for his excellent work at the phosphorimaging facility. This work was supported by NIH grants HL18645 and HL30946.

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