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Concentration-dependent Effects of Peroxovanadium Compound bpV(phen) on PC12 Cell Survival*

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Peroxovanadium compounds are potent insulinomimetic agents and protein tyrosine phosphatase inhibitors. In this study, the potential toxicity of peroxovanadium complex bpV(phen) on rat pheochromocytoma PC12 cells was examined, and the mechanism by which this compound influences cell survival and/or death was explored. BpV(phen) exerted a bimodal effect on PC12 cells: survival-enhancing effect at lower and death-inducing effect at higher micromolar concentrations. 1 and 3 μmol dm^{-3} bpV(phen) intensely induced ERK activation. In contrast, 10 and 100 μmol dm^{-3} bpV(phen) stimulated strong and sustained JNK and p38 MAPK activation as well as caspase-3 activation that preceded bpV(phen)-induced apoptotic cell death. It is suggested that bpV(phen) might exert its action on PC12 cell survival by modulation of MAPKs and caspase-3 activation.

Keywords
peroxovanadium compounds
MAPK
caspase
apoptosis
PC12 cells

INTRODUCTION

Vanadium is a dietary trace element suggested to be essential for higher animals. In the late 1970s and the early 1980s, it was demonstrated that vanadate (V^{5+}) solutions produce insulin-like effects in rat diaphragms and isolated adipocytes *in vitro*.[1] In the following years, the insulinomimetic actions of vanadium salts were examined in a large variety of insulin-responsive cells and tissues and it turned out that they mimic most of the known metabolic effects of insulin. Vanadium salts activated hexose transport in adipose and muscle tissues, activated glycogenesis and inhibited glycogenolysis in liver.[2] Hepatic gluconeogenesis was suppressed by lowering the phosphoenolpyruvate carboxykinase (PEPCK) mRNA levels[3] and by inhibition of glucose-6-phosphatase activity,[4] an event that can also account for the ability of vanadate to arrest glycogen breakdown. Glycolysis in liver was enhanced due to the vanadate-mediated inhibition of fructose 2,6-bisphosphatase[5] and induction of the L-type pyruvate kinase gene.[6] Thus, vanadate inhibits several key metabolic enzyme systems in liver, muscle and adipose tissue, all of which act collectively towards utilizing or storing cell-entered glucose, as well as blocking the effects of hormones that oppose insulin action.

Peroxovanadium compounds are a powerful class of insulinomimetic agents that were discovered when the two insulin-like agents, vanadate and hydrogen peroxide (H_2O_2), were combined and found to be synergistic in their activities to produce insulin-like effects. Peroxovanadium

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compounds resemble the phosphate structure, similar to vanadate species, but the addition of peroxo group(s) sequentially increases their potency as protein tyrosine phosphatase (PTP) inhibitors, presumably by increasing their abilities to irreversibly oxidize the bound thiols.[7,8]

Most data support the inhibition of PTPs and the resultant indirect stimulation of tyrosine phosphorylation as the mechanism by which vanadium compounds promote their insulin-like effects.[9] Although one might suspect that the insulin receptor is involved, the latest studies indicate that the molecular basis of insulinomimetic effects of vanadium or vanadium salts does not involve the insulin receptor tyrosine kinase activity and the subsequent phosphorylation of insulin receptor substrate-1 (IRS-1), but that vanadium salts activate phosphatidylinositol 3-kinase (PI3-K) and mitogen-activated protein kinases (MAPKs).[10–12]

MAPKs are important components in the intracellular regulatory network that transduce extracellular signals to intracellular responses. There are currently four major classes of MAPKs, including extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK), p38 MAPK, and big MAPK (BMK)/ERK5. The functions of ERK3 and ERK7, as well as of the recently identified ERK8, are less understood.[13] A common feature for activation of all MAPK isoforms is the requirement for phosphorylation of both a threonine and a neighbouring tyrosine regulatory site by a specific upstream protein kinase.[14] Once activated, MAPKs phosphorylate other cytoplasmic proteins and translocate from the cytoplasm to the nucleus to regulate the activity of transcription factors, thus modulating expression of different genes. MAPK cascades participate in a diverse array of cellular programs, including cell motility, cell division, proliferation, differentiation, cell survival, and cell death.[13,15,16]

In mammalian cells, the sophisticated interplay between factors that promote or suppress apoptotic cell death results in a complicated regulatory network, which determines the fate of an individual cell as part of its multicellular environment. Members of caspase family are activated in most scenarios of apoptosis. Caspases specifically cleave their substrates after aspartic acid residues and the activity of these proteases depends on the critical cysteine residue within a highly conserved active-site pentapeptide QACRG. In the cell, caspases are synthesized as inactive zymogens, the so called procaspases. So far, 14 different members of this family have been described in mammals, with caspase-11 and caspase-12 only identified in the mouse.[17,18] Because they bring about most of the visible changes that characterize apoptotic cell death, caspases can be thought of as the central executioners of the apoptotic pathway.[19]

The aim of the present study was to examine the potential toxicity of peroxovanadium complex potassium bisperoxo(1,10-phenanthroline)oxovanadate (V) [bpV(phen)] on rat pheochromocytoma PC12 cells, as well as to explore the mechanism by which this compound influences cell survival and/or cell death.

EXPERIMENTAL

Cell Culture

Rat pheochromocytoma PC12 cells[20] were cultured at 37 °C in a 5 % CO_2 atmosphere in RPMI 1640 medium supplemented with 10 % heat-inactivated foetal bovine serum (FBS), 100 IU/ml (≈60 μg/ml) penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B, 290 μg/ml L-glutamine and 2.5 mg/ml sodium hydrogen carbonate. Cells were plated in 6 or 24 well plates, usually at a plating density from 10^5–10^6 cells/ml.

Treatment

After 24 h preincubation, PC12 cells were treated with 1, 3, 10 and 100 μmol dm^{-3} bpV(phen) for 5, 12, 24 or 48 hours. BpV(phen) was provided courtesy of Dr Alan Shaver (Department of Chemistry, McGill University, Montreal, Canada). Stock solution (10 mmol dm^{-3}) was prepared by dissolving bpV(phen) in a phosphate buffered solution (PBS) and protected from light.

MTT Colorimetric Assay

PC12 cells were plated in 24 well plates and treated with bpV(phen) for 24 and 48 hours. At the end of the treatment, cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 4 hours at 37 °C in a humidified atmosphere of 95 % air and 5 % CO_2. Cells were washed twice with PBS, pH = 7.4, and DMSO was added to each well. After 20 minutes, cells were centrifuged at 4 °C at 14000 rpm. The absorbance was measured at 595 nm using a microplate reader.

Lactate Dehydrogenase (LDH) Assay

50 μl of culture supernatant was assayed, using 2 ml of working reagent (61.43 mmol dm^{-3} Tris buffer, 0.20 mmol dm^{-3} NADH, pH = 7.4) and 10 μl of substrate (21.5 mmol dm^{-3} pyruvate). Absorbances were measured at 339 nm every 30 s over a 5-min time period on Pye-Unicam SP8-100 UV/Vis spectrophotometer, and the LDH activity in μmol min^{-1} dm^{-3} was calculated.[21]

Nuclear Staining

Chromatin integrity was examined by staining PC12 cells with Hoechst 33258 (2-[2-(4-hydroxyphenyl)-6-benzimidazole]-6-(1-methyl-4-piperazyl)-benzimidazole-trihydrochloride; bisbenzimide) at a concentration of 1 μg/ml in PBS for 15 min. After rinsing with PBS, chromatin condensation in 300 cells was examined by fluorescence microscopy (Olympus BX50), magnification 1000x.

Whole Cell Lysis

Treated and untreated PC12 cells were washed twice with cold PBS and lysed with 100 μL of ice-cold whole-cell lysis buffer (50 mmol dm^{-3} Tris-HCl pH = 8.0, 137 mmol dm^{-3} NaCl, 1 % Nonident P-40, 10 % glycerol, and »Complete protease inhibitor« cocktail tablet). After 20 minutes, cell lysates were subsequently centrifuged at 4 °C at 14000 rpm and the total protein content was determined by the method of Lowry with bovine serum albumin as the protein standard.[22] Samples were denatured by boiling for 3 minutes with 6x Laemmli sample buffer (0.375 M Tris-HCl pH = 6.8, 12 g/100 ml SDS, 3 % glycerol, 0.2 g/100 ml bromophenol blue, 12 % β-mercaptoethanol in distilled water).

Western Blotting

35 μg of total proteins were loaded for each sample onto a 12 % polyacrylamide gel, usually run at 100 V. Transfer onto nitrocellulose membrane was conducted at 250 mA for 90 minutes. Membranes were blocked for one hour with blocking buffer containing 1 g/100 ml BSA and 1 g/100 ml chicken egg albumin in TBS+T (25 mmol dm^{-3} Tris pH = 7.6, 150 mmol dm^{-3} NaCl, 0.05 % Tween 20). Membranes were probed overnight at 4 °C with anti-JNK-P antibody (Promega) diluted 1:5000 in blocking buffer, anti-ERK-P antibody (Promega) diluted 1:5000 in blocking buffer, anti-p38-P antibody (Promega) diluted 1:2000 in blocking buffer, anti-JNK_1(FL) antibody (Santa Cruz Biotechnology) diluted 1:800 in blocking buffer, anti-ERK_1(C-16) antibody (Santa Cruz Biotechnology) diluted 1:1000 in blocking buffer, anti-p38(C-20) antibody (Santa Cruz Biotechnology) diluted 1:1000 in blocking buffer, or anti-caspase-3(H-277) antibody (Santa Cruz Biotechnology) diluted 1:200 in blocking buffer. A horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences) diluted 1:4000 in 5 g/100 ml low fat milk in TBS+T was utilized to allow detection of the appropriate bands using enhanced chemiluminescence reagent (ECL™ Western Blotting Detection Reagents, Amersham Biosciences) and film (X-OMAT Blue film, Kodak). Membranes were stripped by incubation at 65 °C for half an hour in stripping buffer (100 mmol dm^{-3} β-mercaptoethanol, 2 g/100 ml SDS and 62.5 mmol dm^{-3} Tris-HCl pH = 6.7). For MAPKs, stripping was done separately for each antigen after the analysis of unphosphorylated protein. All experiments were conducted at least three times and representative blots were analyzed using ScionImage software for Windows (Scion Corporation).

Statistical Analysis

Data were analyzed by a one-way analysis of variance (ANOVA) followed by a multiple comparison procedure (Dunnett's test). The level of $P<0.05$ was considered statistically significant for all tests performed. We also performed Levene's test and established homogeneity of variance among all the groups of data assessed. The SPSS computer program for MS Windows, release 6.0, was used for statistical analysis.

RESULTS

Effect of bpV(phen) on PC12 Cell Survival

PC12 cells were treated with 1, 3, 10 and 100 μmol dm^{-3} bpV(phen) in order to assess bpV(phen) toxicity and determine the mode of cell death. Cell viability was evaluated at 24 and 48 hours by three different assays: MTT colorimetric assay was used in order to assess cellular reducing potential (Figure 1a), LDH assay was applied to monitor cellular membrane integrity (Figure 1b) and Hoechst 33258 staining was employed to observe nuclear chromatin integrity (Figure 2).

BpV(phen) cytotoxicity, as measured by different criteria, was concentration-dependent. Both 1 μmol dm^{-3} and 3 μmol dm^{-3} bpV(phen) induced PC12 cell metabo-

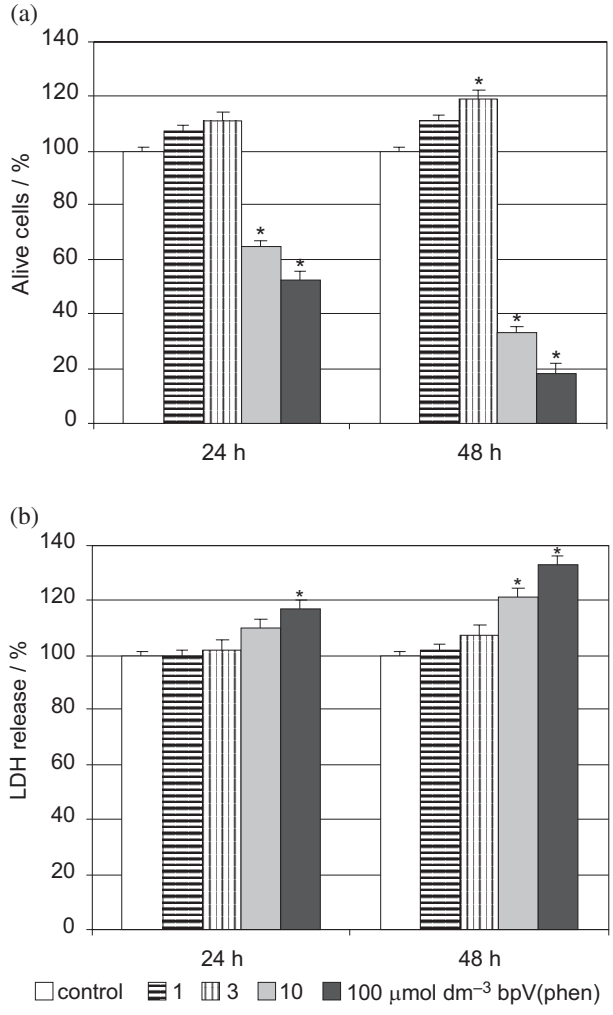

Figure 1. Effects of bpV(phen) on PC12 cell viability. Cells were treated with 1, 3, 10 and 100 μmol dm^{-3} bpV(phen). Cell viability was examined at 24 h and 48 h by MTT reduction assay (a) and LDH release assay (b). Values for untreated control cells were taken as 100 % and values for bpV(phen)-treated cells were taken as the increase and decrease in MTT reduction (a) or increase in LDH release (b), compared to controls. Data represent the mean + SD of six replicates for each experimental condition. Significantly different from control values, $*P<0.05$.

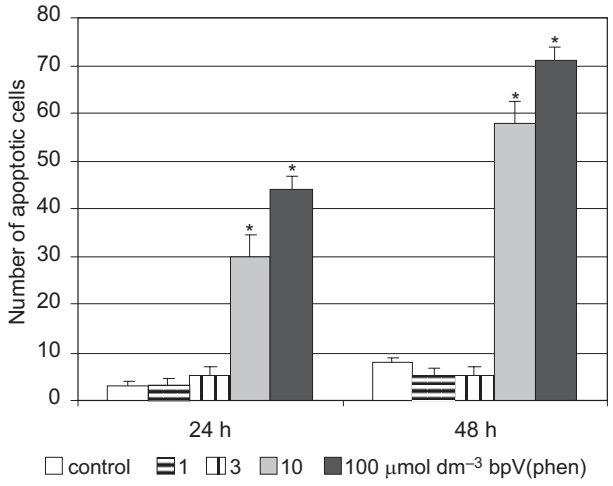

Figure 2. Induction of apoptosis by bpV(phen). PC12 cells were treated with 1, 3, 10 and 100 μmol dm^{-3} bpV(phen) for 24 h and 48 h. Cells were stained with Hoechst 33258 dye and quantification of nuclei with condensed, intensively stained chromatin was performed. Data represent the mean + SD of the number of apoptotic cells in 100 cells examined for each experimental condition. Significantly different from control values, $*P<0.05$.

lic activity, especially at 48 h (Figure 1a). No statistically significant LDH leakage (Figure 1b) or changes of PC12 cells chromatin integrity (Figure 2) could be detected after treatment with lower micromolar bpV(phen) concentrations, as compared to untreated control cells. In contrast, 10 μmol dm^{-3} and 100 μmol dm^{-3} bpV(phen) caused a high inhibition of mitochondrial function and provoked LDH release (Figure 1) after 24 h and even more so after 48 h of treatment. Thus, a decline in cellular metabolic activity occurred before a breakdown of membrane integrity in PC12 cells treated with higher micromolar bpV(phen) concentrations, which is consistent with apoptotic cell death. Furthermore, the same treatment caused chromatin condensation, one of the hallmarks of apoptotic process (Figure 2). Some necrotic cells were observed after incubation with 10 and 100 μmol dm^{-3} bpV(phen). This is in agreement with increased LDH release that could occur due to necrotic cell death or, in a later stage, of apoptosis due to increased permeability of the cell membrane.

BpV(phen) Modulates MAPKs and Caspase-3 Activation

In order to explore the mechanism by which bpV(phen) influences PC12 cell survival and death, we examined MAPKs expression and activation, and procaspase-3 expression by the Western blot analysis after 5, 12 and 24 hours of treatment.

The level of expression of all three MAPKs was not altered by bpV(phen) (data not shown). However, this compound modulated MAPKs activation (Figure 3). ERK phosphorylation was stimulated in cells treated with 1

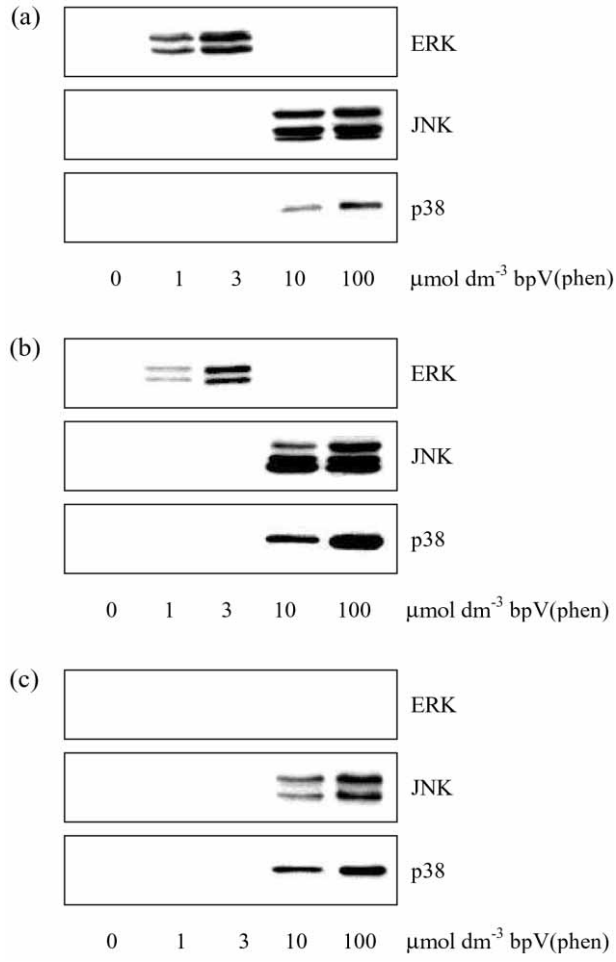

Figure 3. BpV(phen) provokes a concentration-dependent activation of MAPKs. PC12 cells were exposed to 1, 3, 10 and 100 μmol dm^{-3} bpV(phen) for 5 (a), 12 (b) and 24 (c) hours. Western blot analysis was performed as described in the Experimental section. Representative blots for activation of ERK, JNK and p38 MAPK from three independent experiments are shown.

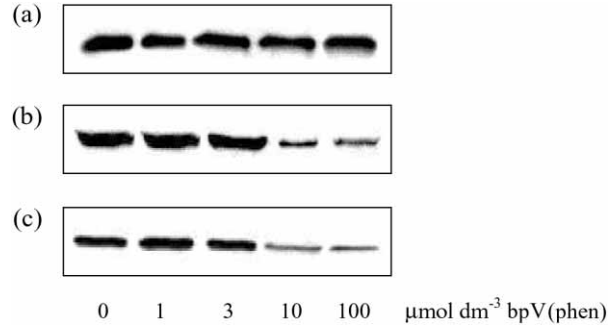

Figure 4. Activation of caspase-3 by bpV(phen). PC12 cells were treated with 1, 3, 10 and 100 μmol dm^{-3} bpV(phen) for 5 (a), 12 (b) and 24 (c) hours. Western blot analysis, using anti-caspase-3(H-277) antibody, was performed. Representative blots for procaspase-3 expression from three independent experiments are shown.

and 3 μmol dm^{-3} bpV(phen) for 5 h and 12 h, and no phospho-JNK or phospho-p38 signal was observed at the same time points examined. In contrast, both 10 μmol

dm^{-3} and 100 μmol dm^{-3} bpV(phen) induced strong and sustained JNK and p38 MAPK activation that could be detected even after 24 h of treatment.

Next, we addressed the question whether the activation of caspase machinery might play a role in mediating PC12 cell death stimulated by higher micromolar bpV(phen) concentrations. 10 and 100 μmol dm^{-3} bpV(phen) stimulated proteolytic processing of procaspase-3 at 12 and 24 h (Figure 4), as shown by decreasing intensity of the procaspase-3 band, suggesting that caspase-3 is activated during the course of bpV(phen)-induced apoptosis.

DISCUSSION

Peroxovanadium compounds are potent insulinomimetic agents and protein tyrosine phosphatase (PTP) inhibitors. In mammals, each cell is simultaneously exposed to multiple signals and must integrate these inputs to choose an appropriate response. By altering the net phosphorylation status of the cell, peroxovanadium complexes could influence cell survival, and the final outcome depends on the cellular context, type of the compound and ancillary ligand within its structure, treatment duration and concentration of the compound used for the treatment.[1,23]

Differentiation of PC12 cells has been observed following incubation with sodium orthovanadate.[24] Furthermore, vanadate exhibits mitogenic properties in SV40-transformed cells.[25] In C3H10T1/3 mouse fibroblasts, peroxovanadates formed *in situ* have been found to lead to proliferation.[26] However, vanadium compounds can also exhibit cytotoxic and antineoplastic effects both *in vitro* and *in vivo*.[1,27,28]

It was shown earlier that vanadium compounds could exert a bimodal effect on cells: survival-enhancing effect at lower and death-inducing effect at higher micromolar concentrations,[29–31] which is in agreement with the results presented in this study. We demonstrated that 1 and 3 μmol dm^{-3} bpV(phen) stimulate PC12 cell metabolic activity. However, 10 and 100 μmol dm^{-3} bpV(phen) caused a high inhibition of mitochondrial function and induced apoptotic cell death.

Vanadate may enhance tyrosine phosphorylation of various endogenous cellular proteins and/or activation of signaling molecules such as mitogen-activated protein kinases (MAPKs). This family includes extracellular signal-regulated kinases (ERKs) that have been linked to cell survival, as well as stress kinases, c-Jun N-terminal kinases (JNKs) and p38 MAPKs, which generally promote inhibition of cell growth and induce apoptosis. This dichotomy, however, is an oversimplification, and the actual roles of each MAPK cascade are highly cell type and context dependent.[15] ERK and JNK/p38 cascade may have opposing effects in differentiated PC12 cells after NGF-withdrawal, and a dynamic balance between the anti-apoptotic ERK pathway and the pro-apoptotic JNK/p38 MAPK pathways appears to be important to determine whether a cell survives or undergoes apoptosis.[32] In this study, we hypothesized that bpV(phen) might exert its action on PC12 cell survival by modulation of MAPKs and/or caspase-3 activation. Lower micromolar bpV(phen) concentrations intensely induced ERK activation, and higher micromolar bpV(phen) concentrations stimulated strong and sustained JNK and p38 MAPK activation. A similar pattern of MAPKs phosphorylation was detected in rat insulinoma RINm5F cells after bpV(phen) treatment.[33] Temporal organization of MAPK activities can play an important role in the generation of specific biological responses. It was demonstrated in PC12 cells that the duration of ERK activation is critical for cellular responses, because transient activation induced cell proliferation and sustained activation caused cell differentiation.[34] Sustained ERK activation is associated with ERK nuclear translocation[35,36] and phosphorylation of transcription factors. Therefore, in contrast to transient activation, sustained activation might have a different effect on gene expression.[34] It has also been reported that duration of JNK activation is a decisive factor for determining cell fate, with transient activation leading to cell proliferation or differentiation, and prolonged JNK activation causing apoptosis.[37–39] Sustained activation of JNK and p38 MAPK has been shown to precede apoptosis of PC12 cells induced by withdrawal of trophic factors.[40] Our results seem to support this notion, since strong and sustained JNK and p38 phosphorylation preceded bpV-(phen)-induced PC12 cell apoptosis. 10 and 100 μmol dm^{-3} bpV(phen) also stimulated cleavage of procaspase-3, suggesting that caspase-3 is activated during the apoptotic process. Caspase-3 activation is one of the hallmarks and commitment steps of programmed cell death. In lymphoid cell lines, pervanadate treatment caused activation of caspases-3, -8 and -9, induction of mitochondrial permeability transition, release of cytochrome c and DNA fragmentation.[41] The interplay between MAPKs and the caspase machinery in the induction of apoptosis was demonstrated previously. It was suggested that JNK signaling plays a major role in activation of the caspase-8 pathway that accounts for vanadate-induced apoptosis of cerebellar granule progenitors.[42] In our study, activation of stress kinases preceded proteolytic processing of procaspase-3. It is possible that bpV(phen)-induced phospho-JNK and/or phospho-p38 MAPK activate caspase-3, which in turn initiate PC12 cell apoptosis. However, due to the complexity and cross-talk among different signaling pathways within a cell, further research is necessary to elucidate this assumption.

CONCLUSIONS

In this study, the effects of peroxovanadium compound bpV(phen) on PC12 cell survival have been examined. BpV(phen) exerted a bimodal effect on PC12 cells: sur-

vival-enhancing effect at lower and death-inducing effect at higher micromolar concentrations. We suggest that the observed bpV(phen) actions might be associated with modulation of MAPKs and caspase-3 activation.

Peroxovanadium compounds are potent insulinomimetic agents and PTP inhibitors. Stability and potency of these complexes renders them attractive agents for managing diabetes mellitus. However, the potentially harmful effects of peroxovanadium compounds may argue against their use in clinics. A better understanding of the molecular mechanism underlying vanadate toxicity and the differences in the cellular response to vanadate is required not only for the therapeutic use of peroxovanadium compounds in diabetes but also for their possible use as antineoplastic agents.

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SAŽETAK

Utjecaj različitih koncentracija peroksovanadijevog spoja bpV(phen) na preživljavanje PC12 stanica

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Spojevi peroksovanadija snažni su inzulinomimetički agensi i inhibitori tirozinskih fosfataza. U ovom istraživanju ispitivana je moguća toksičnost peroksovanadijevoga kompleksa bpV(phen) na PC12 stanicama feokromocitoma štakora te način na koji ovaj spoj utječe na preživljavanje i/ili umiranje stanica. BpV(phen) dvojako je djelovao na PC12 stanice: primijenjen u nižim mikromolarnim koncentracijama poticao je preživljavanje stanica, dok su više mikromolarne koncentracije poticale umiranje stanica. 1 i 3 μmol dm^{-3} bpV(phen) snažno je inducirao aktivaciju ERK kinaza. Suprotno tome, 10 i 100 μmol dm^{-3} bpV(phen) stimulirao je snažnu i dugotrajnu aktivaciju JNK i p38 MAPK kinaza te aktivaciju kaspaze-3, što je prethodilo umiranju stanica procesom apoptoze. Pretpostavlja se da bpV(phen) djeluje na preživljavanje PC12 stanica na način da modulira aktivaciju MAPK kinaza i kaspaze-3.