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Relationship Between Phosphatase Activity and Cytotoxic Effect of Two Protein Phosphatase Inhibitors, Okadaic Acid and Pervanadate, on Human Myeloid Leukemia Cell Line

ANA CLAUDIA GALVÃO FREIRE, HIROSHI AOYAMA, MARCELA HAUN and CARMEN VERÍSSIMA FERREIRA*

Departamento de Bioquímica, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), 13083-970 Campinas, São Paulo, Brasil

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Protein phosphatases are signalling molecules that regulate a variety of fundamental cellular processes including cell growth, metabolism and apoptosis. The aim of this work was to correlate the cytotoxicity of pervanadate and okadaic acid on HL60 cells and their effect on the phosphatase obtained from these cells. The cytotoxicity of these protein phosphatase inhibitors was evaluated on HL60 cells using phosphatase activity, protein quantification and MTT reduction as indices. The major phosphatase presents in the cellular extract showed high activity (80%) and affinity (Km = 0.08 mM) to tyrosine phosphate in relation to *p*-nitrophenyl phosphate (pNPP)—(Km = 0.51 mM). Total phosphatase (pNPP) was inhibited in the presence of 10 mM vanadate (98%), 200 μM pervanadate (95%) and 100 μM p-chloromercuribenzoate (80%) but okadaic acid caused a slight increase in enzyme activity (25%). When the HL60 cells were treated with the phosphatase inhibitors (pervanadate and okadaic acid) for 24 hours, only 20% residual activity was observed in presence of 200 μM pervanadate, whereas in the presence of okadaic acid this inhibitory effect was not observed. However, in respect to mitochondrial function, cell viability decreased about 80% in the presence of 100 nM okadaic acid. The total protein content was decreased 25% when the cells were treated with 100 nM okadaic acid in combination with 200 μM pervanadate. Our results suggest that both phosphatase inhibitors presented different mechanisms of action on HL60 cells. However, their effect on the cell redox status have to be considered.

Keywords: Pervanadate; Okadaic acid; Cytotoxicity; HL60; Protein phosphatase

INTRODUCTION

A variety of cellular functions including gene transcription, cell differentiation, contractility, neurotransmission, and memory regulation involve phosphorylation of proteins which is dependent upon the relative activity of protein kinases and phosphatases.[1] Given the importance of protein phosphorylation in the context of cell function, abnormal protein phosphatase activity has been implicated in diseases such as cancer, diabetes and inflammation.[2–4]

Protein phosphatases are classified according to their substrate specificity, dependence upon metal ions for activity, and sensitivity to inhibitory or activating agents. This defines two families of protein phosphatases: serine/threonine phosphatases (PP1, PP2A, PP2B, PP2C, PP4, PP5 and PP6) and tyrosine phosphatases. Protein tyrosine phosphatases (PTPs) are a structurally diverse family, comprised of receptors (with the ability to transmit signals directly across the membrane) and cytoplasmic enzymes (which act positively and negatively in the control of cell function). Protein tyrosine phosphatases can be divided into 3 groups: tyrosine specific phosphatases, dual and low molecular weight phosphatases.[1,5] The marine toxin (okadaic acid) is a polyether fatty acid that has been reported to cause tumour promotion and reversibly inhibit serine-threonine phosphatase 1 and 2A.[6] This toxin is the most potent inhibitor of PP1 and PP2A and is now regarded as a standard reagent for biological studies on protein phosphatases.[7] Pervanadate is a typical inhibitor of protein tyrosine phosphatase, presumably because of its ability to oxidize thiol groups irreversibly.[8]

Human myeloid leukemia cell line (HL60) has been used as tool for studying the cytotoxic effect of different drugs, differentiation and cell death

*Corresponding author. Fax: +55-19-3788-6129. E-mail: carmenv@unicamp.br

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(apoptosis). Recently, some authors have reported that protein phosphatases may also be involved in this process.[9]

Cytotoxicity may be defined as the measure of the potencial toxicity caused by a compound that, depending on the concentration and exposure time, disrupts cell homeostasis. The deleterious effects (cellular damage) usually compromise cell viability altering morphological and/or metabolic integrity.[10]

The aim of the present work was to investigate the relationship between the phosphatase inhibitory activity and cytotoxicity of pervanadate and okadaic acid, two inhibitors of protein phosphatases, on HL60 cells. Total phosphatase of HL60 cells was characterized by the affinity of substrate and potential inhibitors. These studies were important, since there are no reports of the kinetic properties of these enzyme, which are essential for studing the effects of potential inhibitors. Cell viability was assessed by three parameters: reduction of 3-(4, 5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) that assesses mitochondrial function through the activity of succinate dehydrogenase;[11] protein content that provides an index of the total cell number based on a determination of the cellular macromolecule content[12,13] and phosphatase activity, evaluating cell metabolism in relation to dephosphorylation reactions.[14]

MATERIALS AND METHODS

Materials

HL60 cells were generously supplied by Drs Rui Curi and Valdemir Vieira Colleone from the University of São Paulo (São Paulo, Brazil). The phosphatases inhibitors (vanadate and okadaic acid) and other chemicals were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A).

Methods

Enzyme Preparation

The cells (1×10^6 cells/mL) were washed twice with saline solution and lysed with 0.001 M acetate buffer (pH 5.5). After resting for 10 min, the phosphatase activity[13] was determined.

Phosphatase Activity Assay

Enzyme activity was determined by two methods, depending on the substrate utilized:

pNP Quantification

The reaction medium (final volume of 1.0 mL) contained 100 mM acetate buffer pH 5.5, 5 mM *p*-nitrophenyl phosphate (pNPP) and the enzyme (0.508 μmol/min/mg). After incubation for 30 min at 37°C, the reaction was stopped by addition of 1 M NaOH 1.0 mL. The *p*-nitrophenol (pNP) released was measured at 405 nm.[13]

Phosphate Quantification

When other substrates were used (flavine mononucleotide, tyrosine phosphate, threonine phosphate and serine phosphate), the enzyme activity was determined by measuring phosphate release according to Lowry and Lopez's method.[15]

Preparation of Pervanadate Solution

Pervanadate was prepared by mixing equal volumes of 1 mM hydrogen peroxide and 1 mM sodium metavanadate and incubating the solution at room temperature for 20 min.[16] The solution was then diluted in RPMI (Roswell Park Memorial Institute) medium to the required concentration and used within 30 min.

Cell Culture

HL60 cells were routinely grown in suspension in RPMI medium containing glutamine 0.200 g/L, antibiotics (100 IU penicillin/mL, 100 μg streptomycin/mL) and supplemented with 10% heat-inactivated fetal bovine serum, in a 5% CO_2 humidified atmosphere at 37°C. For the MTT, protein quantification and phosphatase activity assays, 24-well tissue culture plates were inoculated with 3×10^5 cells/ml and incubated at 37°C.[9] After 72 h the cells were treated with pervanadate and okadaic acid, in concentrations up to 200 μM and 100 nM, respectively.

MTT Assay

The medium containing pervanadate and okadaic acid was removed and 1 mL of MTT solution (0.5 mg MTT/mL of culture medium) was added to each well. After incubation for 4 h at 37°C, the medium was removed and the formazan solubilized in 1 mL of ethanol. The plate was shaken for 5 min on a plate shaker and the absorbance then measured at 570 nm.[11]

Protein Phosphatase Assay

The enzyme was obtained after lyse of the cells with 0.1 mM acetate buffer pH 5.5. The reaction mixture (final volume, 0.5 mL) contained 100 mM acetate buffer at pH 5.5, 5 mM pNPP and cell extract enzyme. After a 30 min incubation at 37°C, the reaction was stopped by adding 0.5 mL of 1 M NaOH. The amount of pNP released was determined by measuring the absorbance at 405 nm.[13]

Protein Quantification

Protein concentration was determinated according to a modified Lowry's method.[17]

Statistical Evaluation

All experiments were performed in 24-well tissue culture plates, in triplicate and the results shown in the Table I and Figure 2 represent the mean and standard deviation. Statistical significance/variance were determined by Dunnett's test with the level of significance set at $p < 0.05$.

RESULTS

In this work we describe the relationship between the phosphatase activity and cytotoxic effect of the two protein phosphatases inhibitors (Figure 1) on HL60 cells. Pervanadate, a general term for the variety of complexes formed between vanadate and hydrogen peroxide, and when both are present in 1:1 proportion the major structure is as shown in Figure 1.[16] Pervanadate inhibits PTPs through oxidation the SH group present in their active site. Okadaic acid inhibits protein Ser/Thr phosphatase by hydrophobic interactions between the C-4 to C-16 region of this toxin (Figure 1) and specific domains of these enzymes.[18] The major phosphatase presents in the cellular extract showed high activity (80%)—(Table 1) and affinity (Km = 0.08 mM) of tyrosine phosphate in relation to pNPP (Km = 0.51 mM)—(data not shown). The effect of potential inhibitors, using pNPP as substrate, was analyzed (Table 1). The total phosphatase was inhibited in the presence of 10 mM vanadate (98%), 200 μM pervanadate (95%) and 100 μM *p*-chloromercuribenzoate (80%)-($p < .0001$). Okadaic acid caused a slight increase in enzyme activity (25%).

When the HL60 cells were treated with the phosphatase inhibitors for 24 h, only 20% residual activity was observed in presence of 200 μM pervanadate, whereas in the presence of okadaic acid this inhibitory effect was not observed (Figure 2A). However, in relation to the mitochondrial function, the cell viability decreased about 80% in the presence of 100 nM okadaic acid (Figure 2B). As shown in Figure 2C, the total protein content was decreased 25% when the cells were treated with okadaic acid (100 nM) in combination with pervanadate (200 μM).

DISCUSSION

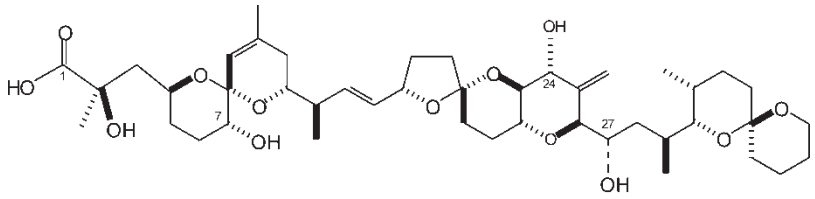
Enzymatic studies that demonstrated high specificity of TyrP, and inhibition by oxidising agents reinforced that the major phosphatase present in the HL60 cell extract could be considered as a protein tyrosine phosphatase (Table 1). Similar results in relation to kinetic properties of PTPs have been commonly reported in the literature.[19,20] All PTPs have SH residues in the active site and, thus, alkylating and oxidising agents of these sulphydryl groups are irreversible inhibitors of this protein phosphatase family.[21] The inhibition presented by pCMB (80%), shows that this phosphatase depends on an essential thiol group for catalysis.[19] The phosphatase of these cells was inhibited completely by *m*-vanadate and pervanadate, reinforcing the predominance of PTP.[22]

Mitochondrial function was significantly affected by okadaic acid, which inhibited 80% the MTT reduction at a concentration of 100 nM (Figure 2B). Some authors reported that this toxin causes the destabilization of this organelle membrane by lipoperoxidation thus, altering energy production.[23,24] Thus, the cytotoxic effect of this toxin on the HL60 cells could be due to its action on the mitochondrial membrane potential.

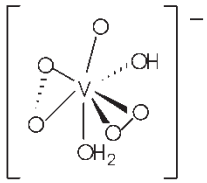
In relation to the action of pervanadate on MTT reduction, (Figure 2B), no significant effect was observed. However, when this result is compared with the cell number, there was a strong stimulation of MTT reduction, because at a concentration of

TABLE I Studies of phosphatase HL60 cells. When the pNPP was utilized as substrate and in absence of compounds the activity was considered as 100%. All experiments were performed in triplicate and the data presented represents the mean ± standard deviation

Assays	Compounds	Concentration	Relative activity (%)
Substrates	pNPP	5 mM	100 (± 6)
	TyrP (tyrosine phosphate)	5 mM	80 (± 1)
	FMN(flavine mononucleotide)	5 mM	35 (± 5)
	ThrP (threonine phosphate)	5 mM	42 (± 2)
	SerP (serine phosphate)	5 mM	37 (± 2)
Inhibitors	Okadaic acid	100 nM	125 (± 1)
	PCMB	0.1 mM	20 (± 1)
	Sodium *m*-vanadate	10 mM	2 (± 1)
	Pervanadate	0.2 mM	5 (± 2)
	NaF	10 mM	60 (± 1)
	SodiumTartrate	10 mM	83 (± 2)
	Phosphate	10 mM	88 (± 2)



Okadaic acid



Pervanadate

FIGURE 1 Chemical structures of protein phosphatase inhibitors.

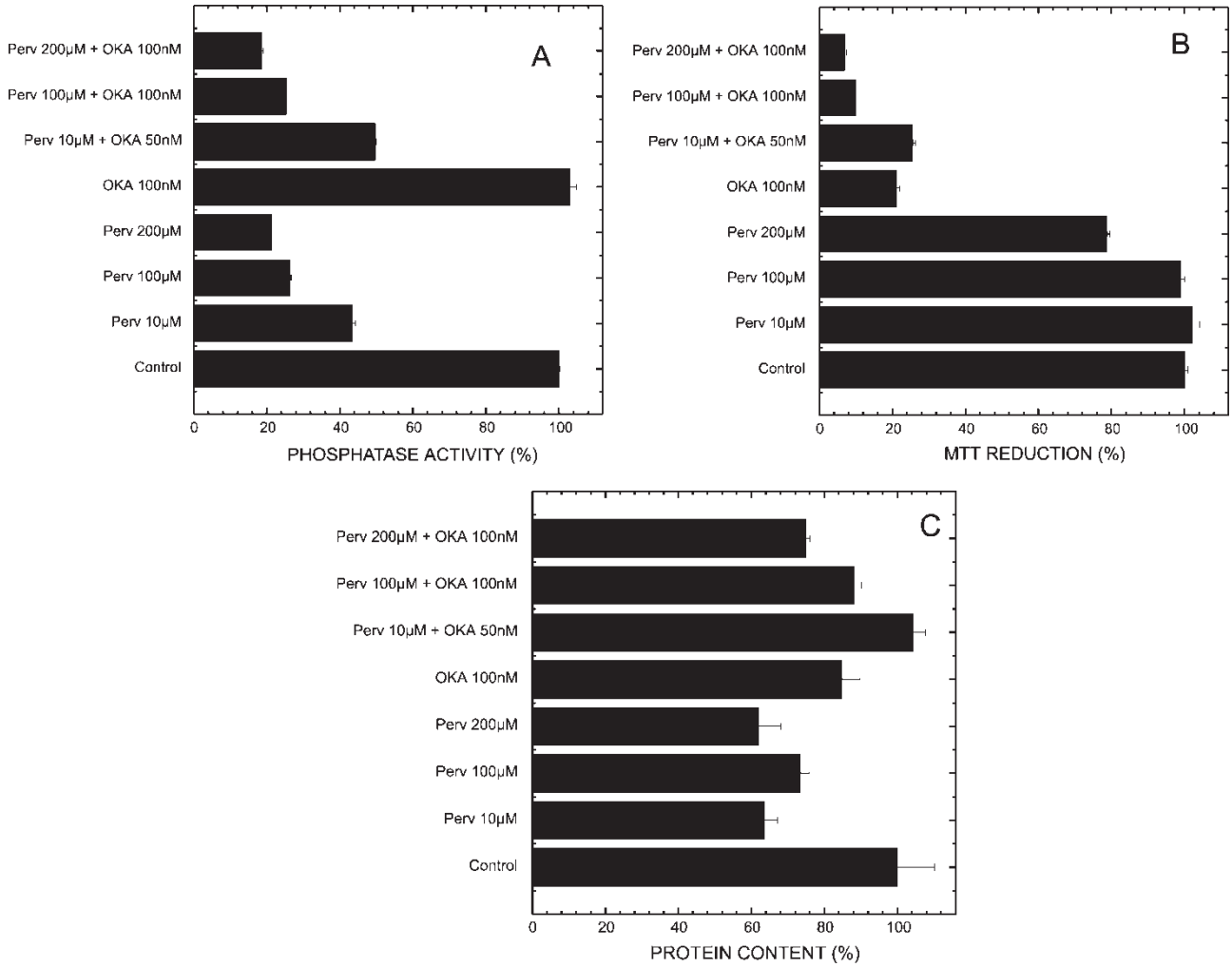


FIGURE 2 Cytotoxicity effect of pervanadate (Perv) and okadaic acid (OKA) on HL60 cells. The histograms show the effects of pervanadate and okadaic acid on the protein phosphatase activity (A), MTT reduction (B) and total protein content (C) in the HL60 cells. In the absence of compounds, all parameters were considered as 100%. All experiments were performed on a plate of 24 wells, and the points shown represent the mean ± standard deviation of at least three experiments run in triplicate ($p < 0.05$).

100 μM the cell number decreased about 30% in relation to non-treated cells. Mosmann (1983) demonstrated that MTT reduction is directly proportional to cell number (non-treated),[11] however in cytotoxic assays sometimes its not observed. This is because a drug can have different cellular targets. However, in the presence of the inhibitor, the redox state of the mitochondria can increase the succinate dehydrogenase expression so leading to an error in the analytical result (mitochondrial function).[25]

On the basis of these results we suggest that the cytotoxicity of pervanadate and okadaic acid may be due an effect on cell signal pathways in which protein tyrosine phosphatases are involved.[26]

Protein phosphorylation–dephosphorylation is probably the most crucial chemical reaction taking place in living organisms and is the basis for the control of diverse biological events, such as metabolism regulation, gene expression, cell division and differentiation.[5] Reversible protein phosphorylation is catalyzed by the opposing and dynamic action of protein kinases and phosphatases. Given the importance of protein phosphorylation in the context of cell function, abnormal protein phosphatase activity has been implicated in several diseases e.g. cancer.[1] The role of tyrosine phosphorylation in mitogenic signaling is well documented, and one would predict that vanadate and pervanadate may act as growth stimulators. Kawakami and colleagues[25] reported that vanadate in the presence of hydrogen peroxide promoted enhancement of tyrosine phosphorylation of various endogenous cellular proteins and/or activation of signaling molecules such as phosphatidylinositol-3-kinase and mitogen-activated protein kinase. At the same time, however, a number of reports indicate that vanadate may inhibit cell proliferation (inhibiting for example, cdc25), and vanadium compounds have been proposed as potential chemotherapeutic agents to inhibit tumor cell growth.[26–28]

In conclusion, inhibition and cytotoxicity studies of protein inhibitors on HL60 cells demonstrated that phosphatase can be used as an additional parameter for cell viability evaluation as recently demonstrated by our research group.[13] In this specific case, an overall analysis of the results indicated that the protein tyrosine phosphatase was the major phosphatase affected by pervanadate. Okadaic acid did not affect the phosphatase activity of HL60 cells treated with this compound, or modify the effect of pervanadate. However, among the parameters analyzed, MTT reduction was significantly affect by this toxin.

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References

[1] Harrison, S., Page, C.P. and Spina, D. (1999) *Gen. Pharmacol.* **32**, 287–298.
[2] Cantley, L.C., Auger, K.R., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R. and Soltoff, S. (1991) *Cell* **64**, 281–302.
[3] Taylor, S.I., Cama, A., Accili, D., Barbetti, R., Quon, M.J., Sierra, M., Suzuli, Y., Koller, E., Levy Toledano, R. and Wertheimer, E. (1992) *Endocrinol. Rev.* **13**, 566–595.
[4] Parnetti, L., Senin, U. and Meccoci, P. (1997) *Drugs* **53**, 752–768.
[5] Jia, Z. (1997) *Biochem. Cell Biol.* **75**, 17–26.
[6] Mayer, A.M.S., Choudhry, M.A., Sayeed, M.M. and Spitzer, J.A. (1997) *Pharmacol. Lett.* **61**, 199–204.
[7] Konoki, K., Sugiyama, N., Murata, M., Tachibana, K. and Hatanaka, Y. (2000) *Tetrahedron* **56**, 9003–9014.
[8] Tsiani, E. and Fantus, I.G. (1997) *Trends Endocrinol. Metabol.* **8**, 51–58.
[9] Uzunoglu, S., Uslu, R., Tobu, M., Saydam, G., Terzioglu, E., Buyukkececi, F. and Omay, S.B. (1999) *Leuk. Res.* **23**, 507–512.
[10] Groth, T., Falck, P. and Miethke, R.R. (1995) *ATLA* **23**, 790–799.
[11] Mosmann, T. (1983) *J. Immunol. Meth.* **65**, 55–63.
[12] Cingi, M.R., Angelis, I., Fortunati, E., Reggiani, D., Bianchi, V., Tiozzo, R. and Zucco, F. (1991) *Toxic. In Vitro* **5**, 119–125.
[13] Freire, A.G., Melo, P.S., Haun, M., Dúran, N., Aoyama, H. and Ferreira, C.V. (2003) *Planta Medica* **69**, 67–69.
[14] Aoyama, H., Melo, P.S., Granjeiro, P.A., Haun, M. and Ferreira, C.V. (2000) *Pharm. Pharmacol. Comm.* **6**, 331–334.
[15] Lowry, O.H. and Lopez, J.A. (1945) *J. Biol. Chem.* **162**, 421–424.
[16] Huyer, G., Liu, S., Kelly, J., Moffat, J., Payette, P., Kennedy, B., Tsaprailis, G., Gresser, M.J. and Ramachandran, C. (1997) *J. Biol. Chem.* **10**, 843–851.
[17] Hartree, E.F. (1972) *Anal. Biochem.* **48**, 422–427.
[18] Maynes, J.T., Bateman, K.S., Cherney, M.M., Das, A.K., Luu, H.A., Holmes, C.F.B. and James, M.N.G. (2001) *J. Biol. Chem.* **276**, 44078–44882.
[19] Cuncic, C., Desmarais, S., Detich, N. and Tracey, A.S. (1999) *Biochem. Pharmacol.* **58**, 1859–1867.
[20] Tracey, A.S. (2000) *J. Inorg. Biochem.* **80**, 11–16.
[21] Zhang, Z.Y. and Dixon, J.E. (1993) *Biochemistry* **32**, 9340–9345.
[22] Enz, A. and Pombo Villar, E. (1997) *Biochem. Pharmacol.* **54**, 321–323.
[23] Cortizo, A.M., Bruzzone, L., Molinuevo, S. and Etcheverry, S.B. (2000) *Toxicology* **147**, 89–99.
[24] Leira, F., Vieites, J.M., Vieytes, M.R. and Botana, L.M. (2001) *Toxicol. in vitro* **15**, 199–208.
[25] Penta, J.S., Johnson, F.M., Wachsman, J.T. and Copeland, W.C. (2001) *Mut. Res.* **488**, 119–133.
[26] Kawakami, N., Shimohama, S., Hayakawa, T., Sumida, Y. and Fujimoto, S. (1996) *Biochim. Biophys. Acta* **1314**, 167–174.
[27] Bernier, L. and Wang, E. (1996) *Exp. Gerontol.* **31**, 13–19.
[28] Zolnierowicz, S. (2000) *Biochem. Pharmacol.* **60**, 1225–1235.