Inhibitors of phosphoprotein phosphatases 1 and 2A cause activation of a 53 kDa protein kinase accompanying the apoptotic response of breast cancer cells

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Received 5 May 1997; revised version received 22 May 1997

Abstract Treatment of MCF-7 breast cancer cells with 50 nM okadaic acid triggers an apoptotic response which is accompanied by a 7-fold increase in the activity of a protein kinase with a relative molecular mass of 53 kDa. The activity of the kinase was stimulated by cell treatment with inhibitors of phosphoprotein phosphatase 1 and 2A, but not by stressing conditions. Okadaic acid-induced stimulation of the 53 kDa protein kinase was not abolished by coincubation of cells with cycloheximide. We conclude that stimulation of the 53 kDa protein kinase by inhibitors of phosphoprotein phosphatases involves pre-existing molecular components whose activity depends on the phosphorylation state of serine/threonine residues.

Key words: Okadaic acid; Phosphoprotein phosphatase; Protein kinase; Apoptosis; Breast cancer

1. Introduction

Protein phosphorylation represents a key molecular mechanism involved in the control of cell differentiation and proliferation [1,2]. The role played by protein phosphorylation in cell death, instead, remains largely undetermined.

The strategies devised to evaluate the role of phosphorylation of individual proteins in cell functioning often involve compounds which may alter either the kinase(s) responsible for the addition of phosphate to specific amino acid residues, or the phosphoprotein phosphatases catalyzing the opposite reaction. Okadaic acid (OA) is a biotoxin of algal origin [3] which binds with high affinity, and then inhibits phosphoprotein phosphatases (PP) 1 and 2A [2,4–6]. This toxin has been shown to induce apoptosis in several experimental systems, but the molecular events involved in the process have not been clarified yet [7–10].

In this report, we show that activation of a 53 kDa protein kinase accompanies breast cancer cell death induced by inhibitors of PP1 and PP2A.

2. Materials and methods

2.1. Materials

Autoradiography films (Reflection), were purchased from DuPont NEN. [γ-^{32}P]ATP (>5000 Ci/mmol) was from Amersham. Tissue culture media and reagents were obtained from Life Technologies. Okadaic acid, calyculin A, and 1-*Nor*-okadaone were from Alexis Corporation (LC Laboratories). Prestained molecular mass markers and myelin basic protein (MBP) were obtained from Sigma. All other reagents were of analytical grade.

2.2. Cell culture conditions and treatments

MCF-7 cells had been obtained from Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia (Brescia, Italy). Cells were grown in 5% carbon dioxide in air at 37°C, in 90 mm diameter Petri dishes, with a culture medium composed of Dulbecco's modified Eagle medium containing 1% nonessential amino acids and 10% foetal calf serum, as previously described [11].

Stock solutions (50 μM) of okadaic acid, calyculin A, and 1-*Nor*-okadaone were prepared by dissolving the compounds in absolute ethanol, and were stored in glass vials protected from light at −20°C. Cell treatments were carried out using dishes near confluency and by addition of stock solutions of compounds directly to culture medium. Parallel dishes received vehicle to obtain control cell samples.

The determination of cell density of individual samples was made by measuring the DNA content in total cell lysates, using the procedure of Labarca and Paigen [12].

2.3. Fractionation of proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Cells were washed three times with 20 mM phosphate buffer, pH 7.4, 0.15 M NaCl (PBS), and were lysed by dispersion in 20 mM phosphate buffer, pH 7.4, 0.15 M NaCl, 1% (v/v) NP-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 50 mM NaF, 10 mM $Na_4P_2O_7$, 0.1 mg/ml phenylmethylsulphonyl fluoride, 1 mM Na_3VO_4, 30 μg/ml aprotinin, and by two 10 s bursts of vortexing. Cytosoluble extracts were then obtained by centrifugation for 30 min at 16000×*g*. The supernatants of this centrifugation were then brought to 2% SDS and 5% β-mercaptoethanol, and proteins were fractionated by SDS-PAGE according to Laemmli [13], using a 10% separating gel and a 3% stacking gel. Molecular mass markers were: α_2-macroglobulin subunit, 180 kDa; β-galactosidase subunit, 116 kDa; fructose-6-phosphate kinase subunit, 84 kDa; pyruvate kinase subunit, 56 kDa; fumarase subunit, 48.5 kDa; lactic dehydrogenase subunit, 36.5 kDa; triosephosphate isomerase subunit, 26.6 kDa.

2.4. In gel kinase assay

Protein kinase activity after fractionation of proteins by SDS-PAGE was detected by the procedure of Kameshita and Fujisawa [14], using separating gels including 0.1 mg myelin basic protein/ml gel. Phosphorylated proteins were detected by autoradiography of dried gels. When quantification of the 53 kDa protein kinase activity was carried out, portions of the dried gel corresponding to the 53 kDa band were excised, and were used for direct measurement of radioactivity. The coefficients of variation of our estimates were lower than 15%.

3. Results and discussion

Preliminary experiments were carried out to check the effect of OA in our experimental system, and we could confirm that OA treatment of MCF-7 cells leads to an apoptotic response [7,8], including a dose- and time-dependent inhibition of cell proliferation, which, as judged by thymidine incorporation, cell detachment from substratum, and measurements of cell

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PII S0014-5793(97)00659-5

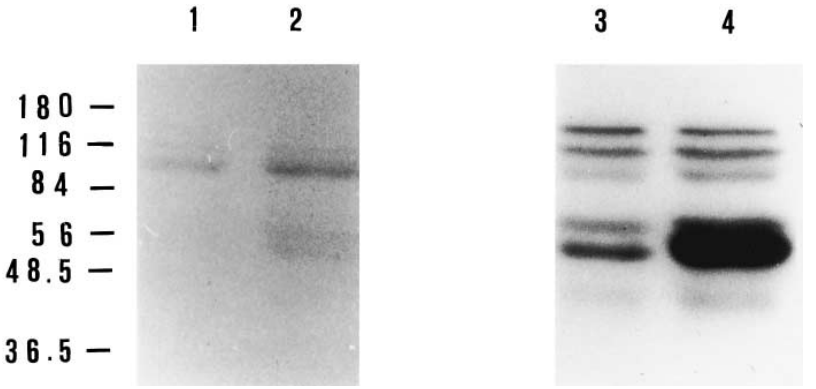

Fig. 1. In gel kinase assay of components from MCF-7 cells. Extracts were prepared from control cells (lanes 1 and 3) and cells which had been treated with 50 nM okadaic acid (lanes 2 and 4) for 24 h at 37°C. Equal amounts of protein were loaded onto each lane and were separated by SDS-PAGE in gels containing no substrate (lanes 1 and 2) or 0.1 mg/ml myelin basic protein (lanes 3 and 4), before being renatured and used for assay of protein kinase activity, as described in Section 2. The positions of marker proteins running in parallel lanes have been indicated. The exposure of lanes 1 and 2 was double than that of lanes 3 and 4, in order to allow visualization of the low levels of autophosphorylation of fractionated proteins, detectable in the absence of MBP.

content of culture dishes, is maximal after a 24 h exposure to 50 nM OA (not shown). OA has been also shown to activate mitogen activated protein kinase/extracellular signal regulated kinase (MAPK/ERK) isoforms in some experimental systems [9,15–18]. In the light of the fact that both p44 and p42 ERKs represent protein kinases involved in the control of cell proliferation [19], we chose to use MCF-7 cell treatment for 24 h with 50 nM OA, to evaluate whether it might affect the activity of p42/p44 ERKs. To this end we used an in gel kinase assay using myelin basic protein (MBP) as the substrate [14]. The results we obtained, showed that extracts prepared from both control and OA-treated cells contained protein kinase activities which could be measured when a suitable substrate (MBP) was included in the gel (Fig. 1). Under these conditions, we found that MCF-7 cell treatment with OA induced only a marginal increase of p42/p44 ERK activity (Figs. 1 and 3). As it is apparent from the data shown in Fig. 1B, however, OA treatment of MCF-7 cells resulted in a net increase in the extent of phosphorylation of MBP by a protein kinase with a calculated molecular mass of 53 ± 2.5 kDa ($n = 10$).

We then determined the time-course of the OA-induced stimulation of MBP phosphorylation by in gel kinase assay, and the results of a typical experiment are shown in Fig. 2. Maximal levels of OA-induced stimulation of the 53 kDa protein kinase activity were detected after 12 h of MCF-7 cell treatment with the toxin, and this stimulation was maintained in the next 12 h (Fig. 2). According to our estimates, the levels of the 53 kDa, OA-stimulated protein kinase (OSK) detected after a 24 h treatment of MCF-7 cells, were about 7-fold (6.9 ± 1.0, $n = 11$) higher than those found in control cells.

Cell treatment with OA has been shown to cause activation of a 55 kDa protein kinase [20], which is a member of the c-Jun NH_2-terminal kinase/stress-activated protein kinase family (JNK/SAPK, Refs. [21–23]). Although MBP does not represent a good substrate for these kinases, as assessed by in gel kinase assay [20–22], we thought it was important to ascertain whether OSK might represent some particular isoform of SAPK. To this end, we compared MBP phosphorylation, using extracts prepared from MCF-7 cells which had been either treated with OA, or subjected to classical stressing conditions, such as heat shock and inhibition of protein synthesis [21,23]. The results we obtained (Fig. 3), showed that increased 53 kDa kinase activity could be detected after OA, but not heat (lane 3) or cycloheximide (lane 4) treatment of MCF-7 cells.

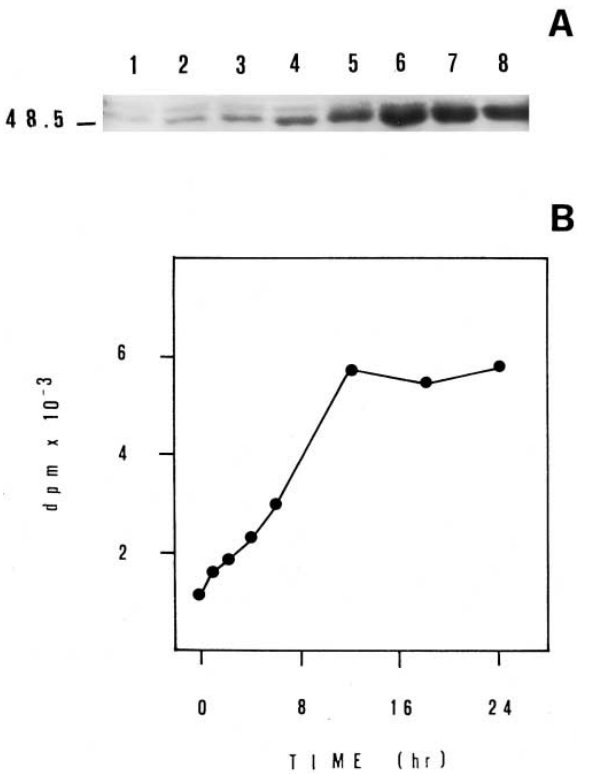

Fig. 2. Time-course of the effect of okadaic acid on the activity of the 53 kDa protein kinase obtained from MCF-7 cells. (A) Cells were treated with 50 nM OA for 0 (lane 1), 1 (lane 2), 2 (lane 3), 4 (lane 4), 6 (lane 5), 12 (lane 6), 18 (lane 7), and 24 (lane 8) h at 37°C, before being used to prepare cell extracts. Equal amounts of protein were fractionated by SDS-PAGE with a separating gel containing MBP, and the gel was used for in gel kinase assay and autoradiography, as described in Section 2. The electrophoretic mobility of pre-stained fumarase marker (48.5 kDa) running in a parallel lane has been indicated. (B) The bands corresponding to the 53 kDa protein kinase of the gel shown in (A) were excised and used for direct measurement of the radioactivity incorporated in the MBP substrate.

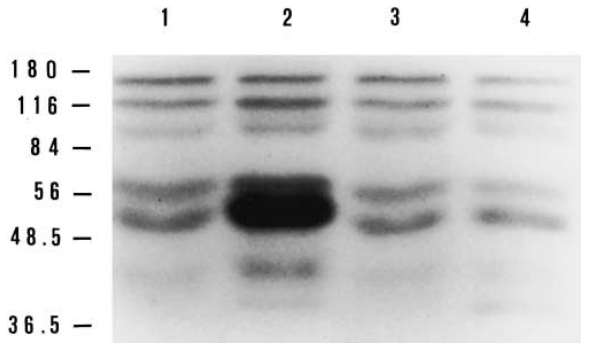

Fig. 3. Effect of MCF-7 cell stimulation by stressing conditions on the levels of 53 kDa protein kinase activity. Cells were treated with either 50 nM OA (lane 2), or 1 mM cycloheximide (lane 4), or vehicle (lanes 1 and 3) for either 24 h at 37°C (lanes 1, 2, and 4), or 1 h at 43°C (lane 3), before being used to prepare cells extracts, which were fractionated by SDS-PAGE with a separating gel containing MBP. At the end of electrophoresis, the gel was used for in gel kinase assay, as described in Section 2. The positions of marker proteins running in a parallel lane have been indicated.

These results prompted us to check whether increased 53 kDa kinase activity could be selectively triggered by inhibitors of serine/threonine phosphoprotein phosphatases. MCF-7 cells were then treated with either OA or calyculin A, or with 1-*Nor*-okadaone, which is an inactive metabolite of OA [24], and the 53 kDa kinase activity was determined by in gel kinase assay. As it is shown in Fig. 4, only OA and calyculin A, which are potent inhibitors of PP1 and PP2A [2,4–6] could cause enhancement of MBP phosphorylation by the 53 kDa kinase, indicating that high levels of OSK activity depend on maintenance of some component(s) in a phosphorylated state.

If the time-course of OA stimulation of the 53 kDa protein kinase is considered (Fig. 2), the 10–12 h needed to reach maximal levels of activity pose the question on whether the phenomenon might represent a secondary response involving neosynthesis of components mediating the effect of OA. We then ascertained whether OA stimulation of the 53 kDa protein kinase could be also observed under conditions in which protein synthesis is inhibited by at least 95% [25]. The results we obtained (Figs. 3 and 5), showed that a 24 h treatment of MCF-7 cells with 1 mM cycloheximide could depress OSK activity detectable in extracts from both control and OA-treated cells by about 40%, but the stimulation of the 53

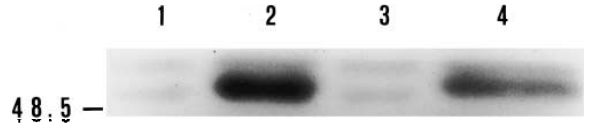

Fig. 4. Effect of MCF-7 cell treatment with inhibitors of serine/threonine phosphoprotein phosphatases on the levels of 53 kDa protein kinase activity. Cells were treated for 24 h at 37°C with either 50 nM OA (lane 2), 50 nM 1-*Nor*-okadaone (lane 3), 5 nM calyculin A (lane 4), or vehicle (lane 1), before being used to prepare cell extracts, which were fractionated by SDS-PAGE with a separating gel containing MBP. At the end of electrophoresis, the gel was used for in gel kinase assay, as described in Section 2. The electrophoretic mobility of pre-stained fumarase (48.5 kDa) marker running in a parallel lane has been indicated. These results have been taken from one experiment, and are representative of those obtained in the two separate experiments we have prerformed.

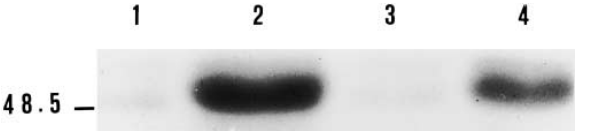

Fig. 5. Effect of inhibition of protein synthesis on the stimulation of 53 kDa protein kinase activity caused by okadaic acid. MCF-7 cells were treated for 24 h at 37°C with either 50 nM OA (lanes 2 and 4), or vehicle (lanes 1 and 3), and in the presence (lanes 3 and 4), or in the absence (lanes 1 and 2) of 1 mM cycloheximide, before being used to prepare cell extracts, which were fractionated by SDS-PAGE with a separating gel containing MBP. At the end of electrophoresis, the gel was used for in gel kinase assay, as described in Section 2. The electrophoretic mobility of pre-stained fumarase (48.5 kDa) marker running in a parallel lane has been indicated. These results have been taken from one experiment, and are representative of those obtained in the three separate experiments we have performed.

kDa kinase activity due to OA was not prevented by blocking protein synthesis, under our experimental conditions (Fig. 5). Furthermore, the same levels of OSK activity were detected in the extracts prepared from cells which had, or had not, been treated with cycloheximide during shorter (8–12 h) incubations with OA (not shown). On the basis of these findings, therefore, it can be concluded that the increase of the 53 kDa protein kinase activity induced by inhibitors of serine/threonine phosphoprotein phosphatases, involves preexisting components, whose functioning is controlled by the phosphorylation state of serine/threonine residues which are themselves substrates of PP1 or PP2A.

Indeed, the 53 kDa protein kinase we have detected might itself represent a substrate of PP1 and/or PP2A. This interpretation, however, can be directly tested after OSK will be available in a pure form. Experiments are in progress to achieve this goal, which will also lead to identification of OSK. In any case, the information available so far allow a preliminary comparative evaluation among protein kinases sharing properties with the 53 kDa kinase we have detected. The molecular mass of OSK, for instance, would indicate that it should not represent one of the protein kinases known to be involved in the control of cell proliferation, and being capable to use MBP as the substrate, such as protein kinase C [26] and MAPK [19,27] isoforms. It seems also unlikely that OSK might represent calmodulin dependent protein kinase II, which can use MBP as the substrate in the kinase assay we have employed in this study [14]. Inclusion of 0.15 mM $CaCl_2$ and 10 μg/ml calmodulin to the mixtures used in our assay, in fact, did not alter the extent of OSK activity detectable in the samples prepared from both control and OA-treated cells (not shown). Furthermore, the property to efficiently use MBP as the substrate, and the lack of activation by heat shock and cycloheximide, would also indicate that OSK should not represent an isoform of the JNK/SAPK family [21–23].

On the basis of the fact that maximal activation of the 53 kDa protein kinase accompanies the apoptotic response of MCF-7 cells to OA treatment, our working hypothesis is that OSK may represent a functional homologue of stress-activated protein kinases. In any case, the identification and a more complete characterization of OSK should be pursued, as its activation accompanies death of breast cancer cells, and then this 53 kDa protein kinase may become a relevant molecular target of therapeutical intervention.

Acknowledgements: This work was supported by a grant from the Italian Consiglio Nazionale delle Ricerche (95.02420.CT04), and by the Italian MURST (quota 60%).

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