Mitotic Arrest and Enhanced Nuclear Protein Phosphorylation in Human Leukemia K562 Cells by Okadaic Acid, a Potent Protein Phosphatase Inhibitor and Tumor Promoter*

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We investigated the effects of the non-phorbol tumor promoter okadaic acid on human leukemia K562 cells. It was found that okadaic acid potently and reversibly inhibited cell growth, with a nearly complete inhibition of thymidine uptake seen at about 10 nM. The cytotoxicity of okadaic acid was characterized by a marked mitotic arrest of the cells exhibiting scattered chromosomes and abnormal anaphase-like structures, a phenomenon distinct from the typical metaphase arrest caused by colchicine. Okadaic acid (10-1,000 nM) greatly stimulated phosphorylation of a number of nuclear proteins in K562 cells. Phosphorylation of many of the same proteins was also stimulated by 12-O-tetradecanoylphorbol-13-O-acetate, a protein kinase C activator. The present findings, consistent with recent reports that okadaic acid is a potent inhibitor of protein phosphatases 1 and 2A (PP1 and PP2A) shown to be essential for normal mitosis, provided evidence for the first time that okadaic acid inhibition of PP1/PP2A resulted in enhanced nuclear protein phosphorylation and subsequent mitotic arrest.

Okadaic acid, a polyether fatty acid and a diarrhetic shellfish poisoning factor isolated from marine sponges (1), is an inhibitor of protein phosphatases PP1 and PP2A (2) and a potent non-phorbol tumor promoter (3). The molecular basis for the tumor-promoting activity of okadaic acid is thought to be due to its inhibition of PP1 and PP2A, two of four major protein phosphatases of mammalian cells (4), thus enabling maintenance of a high phosphorylation level of cellular proteins, an effect that is similarly achieved by TPA, a potent tumor promoting phorbol ester and protein kinase C activator (5). In this regard, the PP1/PP2A family of protein phosphatases might function as tumor suppressors by dephosphorylating cellular proteins. Several aspects of okadaic acid on cellular regulation have been reviewed recently (6, 7).

Biological effects of the toxin include relaxation (8, 9) and contraction (9) of isolated aorta, contraction of isolated tenia coli (10), increased Ca" current in isolated cardiac myocytes (11), increased phosphorylation of cellular proteins in hepatocytes (12) and adipocytes (12), increased generation of Ca"-independent form of Ca"/calmodulin-dependent protein kinase II in cerebellar granule cells due to phosphorylation of the enzyme (15), stimulation of glucose transporter (12) and its phosphorylation in adipocytes (14), stimulation of Na/K/2Cl cotransporter in erythrocytes (15), and activation of protein kinase activity in adipocytes (16). Recent evidence indicates that the cell cycle is regulated by protein phosphorylation/dephosphorylation reactions. A serine/threonine protein kinase p34<sup>cdc2</sup>, the catalytic subunit of maturation promoting factor or growth-associated histone H1 kinase (17), and expression of the cdc2 family of genes have been shown to be essential for eukaryotic cells to progress from G1 to M phase (for example, Refs. 18-20). Phosphorylation of some nuclear proteins (such as histone H1, lamin B, and nucleolin) by p34<sup>cdc2</sup> has been suggested to be responsible for the mitotic events which include disassembly of the nucleus, chromosome condensation, and generation of mitotic spindle (21). PP1 and PP2A have also been shown to be essential for mitosis. It appears, however, that they are likely involved in mitotic progression (22, 23), a role apparently different from that of p34<sup>cdc2</sup> for mitotic initiation or G1 to M phase progression (18-20) as mentioned above. Dephosphorylation by PP1 and PP2A of p34<sup>cdc2</sup> substrates and other unidentified proteins to their interphase levels of phosphorylation would be an important event in mitotic progression after metaphase or for cells to escape from mitosis.

In the present studies, we report that the PP1 and PP2A inhibitor okadaic acid potently inhibited proliferation of human leukemia K562 cells, which was characterized by enhanced nuclear protein phosphorylation and a pronounced mitotic arrest.

EXPERIMENTAL PROCEDURES

Materials—Okadaic acid was a kind gift of Dr. Thomas R. Soderling or purchased from Moaag BioProducts (Honolulu, HI); TPA was from LC Service (Woburn, MA); ALP was from Calbiochem; [3H]thymidine was from ICN Radiochemicals (Irvine, CA); human leukemia cell line K562 was from American Type Culture Collection (Rockville, MD); media and supplies for cell culture were from Gibco; and colchicine and other compounds were from Sigma.

Cellular Studies—K562 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated bovine calf serum, in a humidified incubator at 37 °C in 95% air, 5% CO2. Cells at the mid-log phase were used in all experiments reported herein. [3H]Thymidine uptake was determined essentially as described by Bastian et al. (24). Briefly, cells (0.5-1 x 10<sup>5</sup> ml/well) were incubated with [3H] thymidine (1 µCi) in the culture medium without serum, with or without the agents to be studied, for 1 h at 37°C, and the uptake was stopped by addition of 1 ml of ice-cold 10% trichloroacetic acid. Cells were collected by centrifugation, washed twice with 1 ml each of 5% trichloroacetic acid, and finally dissolved in 0.25 ml of 0.5% deoxycholic acid in 0.1 N NaOH for radioactivity determination. About 5-15% of the total [3H]thymidine was incorporated into cells under the experimental conditions. Cell viability was determined by exclusion of 0.2% trypan blue dye. Cells were stained with Wright-Giemsa stain for microscopic examination and estimation of cell population caught at mitotic phase.

Protein Phosphorylation—K562 cells (2 x 10<sup>5</sup>/ml) in the incubation medium (140 mM KCl, 0.01 mM CaCl<sub>2</sub>, 3 mM MgCl<sub>2</sub>, 10 mM Hepes-
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Na, pH 7.4) were treated with 0.003% saponin for 5 min at 37°C, pelleted by centrifugation, and washed once with 10 ml of the medium. Permeabilized cells were suspended in 1 ml of the medium and aliquots (0.25 ml) of the cell suspension were incubated (in 0.5 ml) for 20 min at 30°C in the presence of 10 μM [γ-32P]ATP (containing about 0.5-1 × 10^6 cpm) and 100 μM CaCl2, with or without 0.1-10 nM okadaic acid, 100 nM TPA, and 30 μM ALP (Fig. 3). Cells were then recovered by centrifugation, suspended in 0.5 ml of ice-cold homogenization buffer (10 mM Tris/HC1, pH 7.4, 240 mM sucrose, 2 mM EGTA, 2.5 mM MgCl2, 10 mM mercaptoethanol, and 1 mM phenylmethanesulfonyl fluoride), and homogenized (20 up-and-down strokes) with a tight-fitted Teflon-glass homogenizer. Aliquots (0.4 ml) of the homogenate were centrifuged at 1,000 × g for 5 min to yield the post-nuclear (supernatant) and crude nuclear (pellet) fractions, and the nuclear fraction was taken up in 0.4 ml of the homogenization buffer. To the fractions were added 0.25 ml of volume of gel sample buffer (25), and aliquots (80 μl) of the samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography as described elsewhere (25). The cell permeabilization procedure described above was predetermined to be optimum because of high cell viability and [γ-32P]ATP uptake and protein phosphorylation. Alternatively, cellular protein phosphorylation was carried out using the native (saponin untreated) K562 cells prelabeled with 32P. (Fig. 4A), according to the procedures we described earlier for HL60 cells (25). Finally, protein phosphorylation was also examined in the cell-free system for comparison. Briefly, K562 cells (2 × 10^7) were homogenized in 1 ml of homogenization buffer and centrifuged to yield the nuclear and post-nuclear fractions. Aliquots (0.25 ml) of the fractions were incubated (in 0.5 ml) for 20 min at 30°C with 0.5 mM CaCl2 (in excess of EGTA), 10 μg/ml of phosphatidylserine, 10 mM MgCl2, and 10 μM [γ-32P]ATP (about 2 × 10^6 cpm), with or without TPA or okadaic acid (Fig. 4B), essentially as described (26).

RESULTS

Effects of okadaic acid on cell growth, indicated by incorporation of [3H]thymidine into DNA, and viability of K562 cells were examined. The toxin was found to markedly inhibit cell growth at 10 and 15 nM, whereas it had no effect at 1 nM okadaic acid for up to 4 days, as indicated. The data presented are the means of triplicate incubations, with experimental errors being less than 5%. Similar results were obtained in two other experiments.

![Fig. 1. Effects of okadaic acid (OA) on K562 cells.](image)

![Fig. 2. Micrographs showing mitotic arrest of K562 cells by okadaic acid (OA) and colchicine (COL).](image)
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TABLE I
Reversibility of okadaic acid inhibition of K562 cell growth
K562 cells (0.5 x 10^5/ml/well) were preincubated for the indicated times without (control group) or with 10 nM okadaic acid (recovery and toxin groups). Cells were then centrifuged, washed once, and resuspended (0.5 x 10^5/ml/well) in the fresh okadaic acid-free medium without (control and recovery groups), or with 10 nM okadaic acid (toxin group), and allowed to grow for an additional 4 days. [3H]Thymidine uptake was determined for 1 h at day 4. The data presented are means ± S.E. of triplicated incubations.

| Preincubation time (h) | [3H]Thymidine uptake | Control | Recovery | Toxin |
|------------------------|-----------------------|---------|----------|-------|
| 0                      | 74 ± 6               | 59 ± 3  | 27 ± 2^a|
| 18                     | 62 ± 4               | 71 ± 1  | 16 ± 1^a|
| 24                     | 68 ± 7               | 69 ± 2  | 26 ± 1^a|

^a Significantly different from the control and recovery groups (p < 0.005).

Fig. 3. Effects of okadaic acid, TPA, and ALP on protein phosphorylation in saponin-permeabilized K562 cells. Cells were incubated for 20 min under the phosphorylation conditions with various concentrations of the agents, as indicated. See "Experimental Procedures" for further details. Similar results were obtained in four other experiments.

We found that 10 nM okadaic acid caused mitotic arrest of K562 cells, which was characterized by chromosome overcondensation and chromatin degeneration (Fig. 2). These observations were surprisingly similar to those of Axton et al. (22) on ganglions from drosophila which has a mutated gene encoding PP1, indicating that inhibition of PP1 and/or PP2A by the toxin and absence of the enzyme itself could lead to a similar end result. The findings appeared to support the contention that PP1/PP2A might be required in inactivating p34^cdc2 and reversing the effects of the kinase after metaphase (22). It was not totally unexpected, therefore, that okadaic acid inhibition of PP1/PP2A could interfere with normal progression of mitosis from metaphase to anaphase and beyond. Clearly, phosphorylation and dephosphorylation of certain proteins, occurring in an orderly manner, are essential for normal mitotic process. It has been reported that reorganization of interphase microtubules as a mitotic spindle in starfish embryos requires both p34^cdc2 and okadaic acid-sensitive PP1/PP2A activities (28). In this regard, it is of interest to investigate whether the toxin-induced mitotic arrest of K562 cells would exhibit abnormal spindle morphology similar to that seen in the drosophila mutant lacking PP1 (22).
Fig. 4. Effects of TPA and okadaic acid on protein phosphorylation in native K562 cells and in subcellular fractions. The native (not saponin-permeabilized) cells prelabeled with $^{32}$P, (A) or the individual nuclear (N) and post-nuclear (PN) fractions (B) were incubated for 20 min under the phosphorylation conditions, with or without 100 nM TPA or 100 nM okadaic acid (OA), as indicated. See "Experimental Procedures" for further details. Similar results were obtained in two other experiments.

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