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Okadaic acid strongly increases gene transcription, mRNA and protein level for the urokinase receptor in human A549 cells

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The specific phosphatase inhibitor, okadaic acid, increases the level of mRNA for the receptor for urokinase-type plasminogen activator (u-PAR) in 8 out of 13 human cell lines. The strongest increase (90-fold) was observed in A549 lung carcinoma cells, in which it was partly traced back to an increased transcription of the u-PAR gene. There was a parallel but less pronounced increase in the u-PAR protein level. These findings indicate that u-PAR gene transcription is regulated by one or more factors that are constitutively phosphorylated and are dephosphorylated by okadaic acid-sensitive phosphatases. A lack of additivity of u-PAR induction by okadaic acid and by the protein kinase C activator, PMA, in the A549 cells suggests that the regulatory factors affected by okadaic acid are phosphorylated by protein kinase C.

Urokinase receptor; Plasminogen activation; Gene regulation; Okadaic acid; Extracellular proteolysis; Growth factor; Signal transduction

1. INTRODUCTION

Generation of the broad-spectrum protease plasmin by urokinase-type plasminogen activator (uPA) is involved in degradation of extracellular matrix proteins in a variety of biological processes requiring tissue destruction or cell migration. The activity of uPA is regulated by two specific and fast-acting plasminogen activator inhibitors, PAI-1 and PAI-2, and by a specific cell-surface uPA receptor (u-PAR) [1–3].

u-PAR was first detected on monocytes and monocyte-like cells by a specific, high-affinity binding of uPA [4] and its zymogen pro-uPA [5]. It has since been observed on a variety of cultured cell lines of neoplastic and non-neoplastic origin [6–9]. We recently purified and characterized human u-PAR [9,10] and cloned a full-length cDNA coding for this receptor [11]. u-PAR is an M_r 55–66,000, single-chain, highly glycosylated protein attached to the plasma membrane by a glycosyl–phosphatidylinositol lipid anchor [12]. It consists of three homologous repeats of which the N-terminal binds uPA [13].

The synthesis of uPA, its inhibitors and u-PAR is controlled by a variety of hormones, growth factors and cytokines [1,14–16]. Recently we found that the tumor promoter PMA and transforming growth factor-β1 (TGF-β1) in certain cell types increase the u-PAR mRNA level and gene transcription [17,18]. We now report that okadaic acid, an inhibitor of serine/threonine phosphatases [19] increases the u-PAR mRNA level in 8 out of 13 human cell lines. The strongest effect is seen in A549 lung carcinoma cells. In this cell line, okadaic acid also induces u-PAR gene transcription and increases the level of receptor protein.

Abbreviations: uPA, urokinase-type plasminogen activator; u-PAR, uPA receptor; ATF, NH_2-terminal fragment (1–135) of uPA; PAI-1, plasminogen activator inhibitor type-1; PAI-2, plasminogen activator inhibitor type-2; PMA, phorbol 12-myristate 13-acetate; TGF-β1, transforming growth factor-β type 1; EGF, epidermal growth factor; DSS, disuccinimidyl suberate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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2. MATERIALS AND METHODS

2.1. *Materials*

Okadaic acid was obtained from Boehringer (Mannheim, Germany). All other materials were purchased as described [12,13,17,18,20].

2.2. *Cell culture*

The human lung carcinoma cell line A549 (American Type Culture Collection (ATCC) CCL 185), the embryonal lung fibroblast cell lines WI-38 (ATCC CCL 75) and Hel 299 (ATCC CCL 137), the larynx epidermal carcinoma cell line HEp-2 (ATCC CCL 23), the epitheloid cervix carcinoma HeLa (ATCC CCL 2), and the human rhabdomyosarcoma cell line RD (ATCC CCL 136) were obtained from Flow laboratories, Irvine, UK. The promyeloid leukemic cell line HL-60 (ATCC CCL 240) and the chronic myelogeneous leukemic cell line K-562 (ATCC CCL 243) were obtained from ATCC, Rockville Pike, MD, USA. The fibrosarcoma cell lines HT-1080 (ATCC CCL 121) and 8387 were obtained from Dr. A. Vaheri, University of Helsinki, Finland. The monocyte-like cell line U937 (ATCC CRL 1593) was obtained from Dr. A. Fattorossi, Rome, Italy). The melanoma cell lines Bowes and MIAI were obtained from Dr. C. Kluft, Leiden, The Netherlands and Dr. J. Zeuthen, Copenhagen, Denmark, respectively.

Approximately 10^6 trypsinized cells were seeded in 15 cm Petri dishes and grown to confluency in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum as described [18], with the exception of the suspension growing U937, HL-60 and K-562

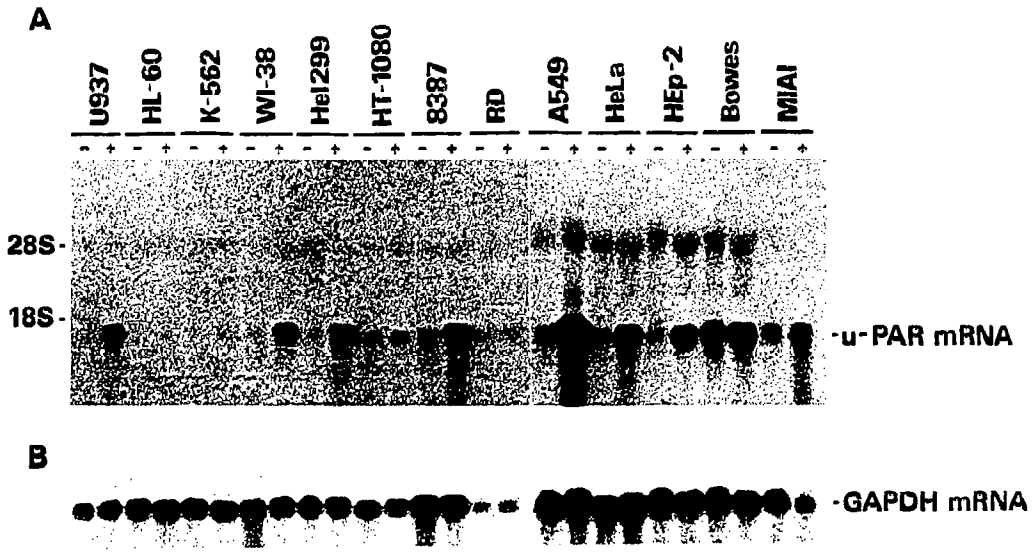

Fig. 1. Northern blot analysis of effect of okadaic acid on u-PAR mRNA level in human cell lines. The indicated cell lines were incubated for 6 h with (+) or without (−) 100 nM okadaic acid and harvested. Their total RNA was isolated. For Northern blot analysis, 30 μg of the total RNA were electrophoresed in 1.5% agarose gels under denaturing conditions and blotted onto a nitrocellulose filter. The membrane was hybridized with a randomly primed ^{32}P-labeled u-PAR cDNA probe (A), and as a control, after stripping, it was rehybridized with a GAPDH cDNA probe (B). The position of the ribosomal RNAs are indicated to the left and the mRNA bands to the right. Ethidium bromide staining of the agarose gel and the blot showed an approximately equal amount of intact RNA in all lanes (data not shown). In this experiment a prolonged exposure of the blot was performed (2 days) in order to obtain detectable u-PAR mRNA signals in the different cell lines.

cell lines which were cultured at a density of approximately 0.5×10^6 cells/ml in RPMI-1640 medium supplemented with 10% fetal calf serum. Fetal calf serum was also present in the medium for these latter cell lines throughout the experiments, while all the adherent cell lines were kept under serum-free conditions during the experiment and through a 48–72 h pre-incubation period. Cells were harvested as described [18].

2.3. *Various procedures*

Total RNA was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction and analyzed by Northern blotting [17]. Chemical cross-linking of u-PAR to ^{125}I-labeled ATF was done on total detergent lysate of the cells [10]. Nuclear transcription assays were performed as described [17].

3. RESULTS

3.1. *Cell-specific expression and regulation of u-PAR mRNA level by okadaic acid in human cell lines*

The responsiveness of the u-PAR mRNA level to okadaic acid was studied in 13 human cell lines by Northern blot analysis with a full-length cDNA probe for u-PAR [11]. Fig. 1 shows that the basal level of u-PAR mRNA varies strongly between the different cell lines. The treatment with okadaic acid resulted in an increased level of u-PAR mRNA in U937, WI-38, Hel 299, 8387, A549, HeLa, HEp-2 and MIAI cells. No effect of okadaic acid was observed in the remaining cell lines.

The strongest response to okadaic acid was observed with the A549 cells which we chose for a more detailed study of the okadaic acid regulation of u-PAR expression. Fig. 2 shows the accumulation of u-PAR mRNA in A549 cells after 6 h incubation with the indicated concentrations of okadaic acid. There was no effect on the GAPDH mRNA level (Fig. 2B), which therefore was used for normalization. A maximal ≈90-fold increase in the u-PAR mRNA level is reached at 100 nM of okadaic acid and a half-maximal effect at ≈20 nM. At an okadaic acid concentration of 200 nM there was a pronounced toxic effect on the cells. Concentrations higher than 100 nM were therefore not tested in the subsequent experiments.

In order to study the kinetics of the induction, the A549 cells were treated with 50 nM okadaic acid for different time periods and analyzed by Northern blotting (Fig. 3). Densitometric scanning of the autoradiograms showed that the u-PAR mRNA level was significantly increased after 1.5 h, reached a maximal 90-fold increase after 16 h of okadaic acid treatment and then remained almost constant for up to 24 h.

3.2. *Effect of okadaic acid on u-PAR gene transcription*

The effect of okadaic acid on transcription of the u-PAR gene was measured with a nuclear transcription (run-on) assay. Nuclei were isolated from A549 cells cultured for 6 h in the presence or absence of 50 nM okadaic acid, followed by incubation with ^{32}P-labelled uridine triphosphate for 40 min. Fig. 3C shows autoradiograms of radiolabelled RNA from these nuclei hybridized to filters containing the indicated cDNA probes. Densitometric scannings of the autoradiograms

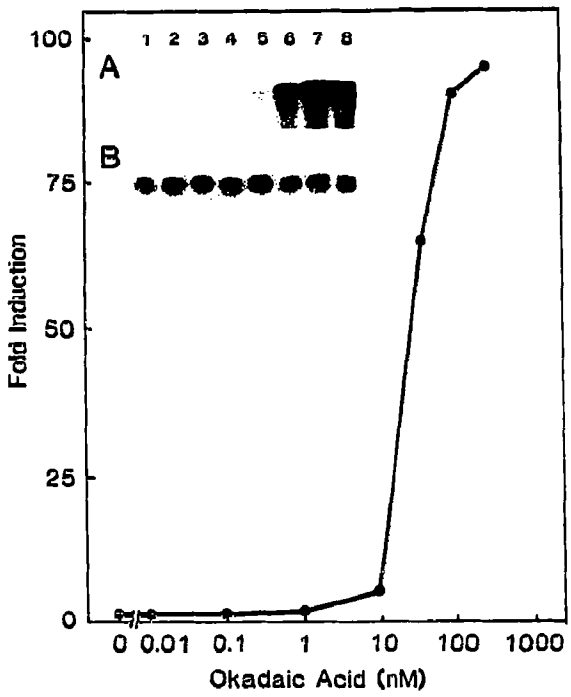

Fig. 2. Dose-dependency of okadaic acid induction of u-PAR mRNA in A549 cells. A549 cells were cultured for 6 h with the indicated concentrations of okadaic acid, harvested and analyzed by Northern blotting as described in the legend to Fig. 1. Hybridizations with the u-PAR cDNA probe is shown in insert A, and re-hybridization of the stripped blot with a GAPDH cDNA probe in insert B. The numbers in the insert indicate non-treated cells (lane 1), and cells treated with okadaic acid in a concentration of 0.01 nM (lane 2), 0.1 nM (lane 3), 1.0 nM (lane 4), 10 nM (lane 5), 50 nM (lane 6), 100 nM (lane 7) and 200 nM (lane 8). The relative amount of u-PAR mRNA at each concentration was estimated by scanning of the Northern membranes hybridized with the u-PAR probe after normalization against the corresponding relative amounts of GAPDH mRNA. The u-PAR mRNA level in the non-treated cells has been set equal to 1 and at each concentration is expressed as fold induction.

showed an 8-fold increase in u-PAR gene transcription while no effect was detected on the level of GAPDH gene transcription.

3.3. *Effect of okadaic acid on u-PAR protein level*

Induction of the u-PAR protein was studied with a sensitive and specific chemical cross-linking technique, in which u-PAR is bound to the amino terminal fragment (ATF) of the u-PA molecule [10]. After treatment of the cells with 50 nM okadaic acid for different time periods, Triton X-114 extracts were cross-linked to ^{125}I-labeled ATF and analyzed by SDS-PAGE and autoradiography. A weak but visible signal of cross-linked ^{125}I-ATF was seen in non-treated A549 cells (Fig. 4). After 3 h of incubation in the presence of okadaic acid, there was an increase in the intensity of the signal, which continued up to 24 h of incubation, after which a small decrease in binding capacity was observed. After the autoradiography the areas of the gel corresponding to u-PAR/ATF complexes were excised and counted in a gamma-counter. This analysis indicated a maximal 15-fold increase in radioactivity after 24 h of okadaic acid treatment (data not shown).

3.4. *Effect of okadaic acid in combination with PMA or TGF-β1 on the u-PAR mRNA level*

At least two signaling pathways are known to involve factors that are dephosphorylated by okadaic acid-sensitive phosphatases, the protein kinase-C pathway and the cAMP-dependent protein kinase pathways [22–26]. Previously, we have found that the protein kinase C activator, PMA, increases the u-PAR mRNA level in A549 cells, while cAMP is without any effect ([18], unpublished result). In order to evaluate whether the okadaic acid effect on the u-PAR mRNA level was mediated by a factor involved in the protein kinase C pathway, we examined the combined effect of okadaic acid and PMA in doses that for each of the compounds had a maximal effect on the u-PAR mRNA level. In a separate experiment the maximally effective dose of PMA was found to be 150 nM with a 6 h incubation period (results not shown). As seen in Fig. 5 there was no additive effect when 100 nM okadaic acid and 150 nM PMA was used in combination. This result would be expected if phosphorylation of the same factor is involved in the action of the two compounds.

The u-PAR mRNA level is also increased by TGF-β1 [18]. The combination of okadaic acid and TGF-β1 resulted in an additive (or even synergistic) effect on the u-PAR mRNA level (Fig. 5). This finding is in agreement with the TGF-β1-dependent signaling pathway not being supposed generally to involve factors influenced by okadaic acid [27].

4. DISCUSSION

In 8 out of 13 human cell lines okadaic acid caused an increase in the level of u-PAR mRNA while no effect on this level was observed in the remaining 5 cell lines. The okadaic acid-responsive cell lines were found both among those of epithelial (A549, HEp-2, HeLa and MIAI), mesenchymal (WI-38, Hel 299 and 8387) and leukemic (U937) origin. The strongest response was seen in A549 cells, lung carcinoma cells, in which the effect was both time- and dose-dependent, the maximal induction observed being approximately 90-fold. This increase was partly traced back to an increased transcription of the u-PAR gene. In the A549 cells there was also an increase in u-PAR protein of maximally 15-fold which was fully accounted for by the increase in the mRNA level.

Okadaic acid is an inhibitor of the serine/threonine-specific protein phosphatases, 1 and 2A [19,26]. The present findings indicate that one or more factors which regulates u-PAR gene expression in the A549 cells (and presumably the other 7 cell lines in which okadaic acid increases the u-PAR mRNA level) are continually being

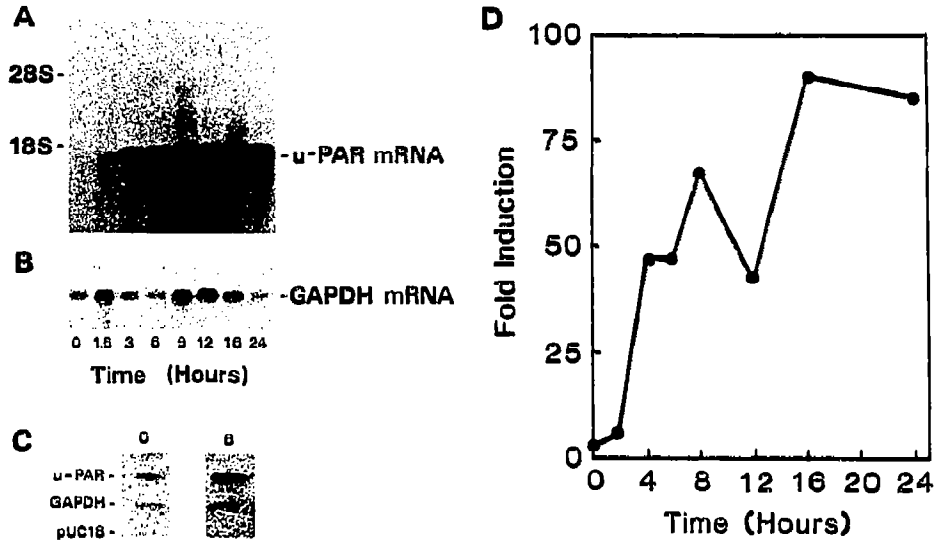

Fig. 3. Northern blot and nuclear transcription analysis of cells treated with okadaic acid for different time periods. Experimental conditions were as described in the legend to Fig. 2, except that the cells were treated with 50 nM okadaic acid for the indicated time periods. The membranes were hybridized with a randomly primed [^{32}P]-labeled u-PAR cDNA probe (A), and, after stripping, re-hybridized with a GAPDH cDNA probe (B). The normalized relative amounts of u-PAR mRNA as determined by densitometric scanning are indicated in (D). In (C) the transcriptional activity of the human u-PAR gene, following stimulation by okadaic acid, was determined by hybridizing nitrocellulose membranes, containing immobilized cDNa probes as indicated, with [^{32}P]-labeled RNA, prepared from nuclei isolated from control cells or cells treated with 50 nM okadaic acid for 6 h. The membranes were exposed for 7 days. pUC18 indicates a control vector cDNA.

phosphorylated and dephosphorylated, the dephosphorylation being catalyzed by okadaic acid-sensitive phosphatases. Okadaic acid will then change the equilibrium towards the phosphorylated form of this factor, which again directly or indirectly enhances u-PAR transcription.

The findings that okadaic acid and PMA each strongly enhance the u-PAR mRNA level in A549 cells, and that the combination of the two compounds in maximally effective doses do not have any additive effect, suggest that the regulating factor(s), which in these cells is affected by okadaic acid, is the same as the factor(s) phosphorylated by protein kinase C after PMA stimulation. An alternative explanation for the lack of additive effect of the two compounds would be that each of them, by independent mechanisms, are able to induce the maximally obtainable transcription rate. This explanation is, however, less likely, because a combination of okadaic acid and TGF-β caused a u-PAR mRNA level which was considerably higher than that obtained with okadaic acid and PMA.

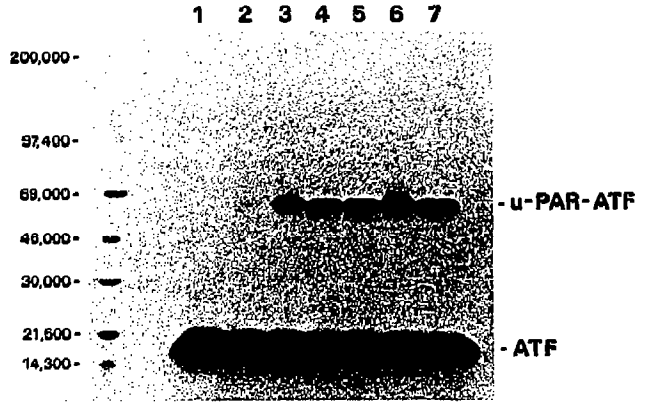

Fig. 4. Time-course of okadaic acid induction of u-PAR protein as detected by chemical cross-linking to ^{125}I-ATF. Non-treated cells (lane 2) and cells treated with 50 nM okadaic acid for 3 h (lane 3), 6 h (lane 4), 12 h (lane 5), 24 h (lane 6) and 48 h (lane 7) were acid-treated to remove endogenous uPA, and detergent extracts were prepared as described in Materials and Methods. Non-diluted extracts and a buffer control sample (lane 1) were incubated with ^{125}I-ATF, cross-linked with DSS and run in a 6–16% SDS-PAGE gradient gel under non-reducing conditions followed by autoradiography. Electrophoretic mobilities of molecular weight standard proteins are indicated on the left. The extracts were prepared from cells used in the experiments described in Fig. 3.

Binding of uPA to its receptor enhances and localizes plasmin formation [2,3] and both uPA and u-PAR appear to play important roles in tissue destruction in normal and pathological conditions, including cancer invasion [1–3,20,21]. In some types of cancer (such as colon adenocarcinomas and squamous skin carcinomas) u-PAR is expressed in cancer cells located at the tumoral–stromal interface of invasive foci but not in cancer cells located in other areas of the tumors ([21], C. Pyke, personal communication). These findings indicate that the u-PAR expression in these cancer cells is regulated by factors in the microenvironment. We have previously shown that different hormones, growth factors and cytokines, such as TGF-β1, EGF and dexamethasone, regulate u-PAR expression in a cell specific manner (17,18], unpublished observations). A clarification of the signal pathways involved in this regulation

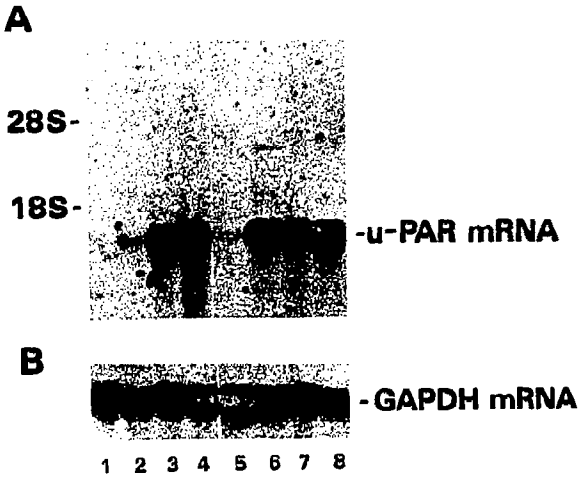

Fig. 5. Northern blot analysis of the effect of okadaic acid in combination with PMA or TGF-β1 on the u-PAR mRNA level in A549 cells. Total RNA was isolated from cells incubated for 6 h with no additions (lanes 1 and 5), with 100 nM okadaic acid (lanes 3 and 7), 5 ng/ml TGF-β1 (lane 2), a combination of 5 ng/ml TGF-β1 and 100 nM okadaic acid (lane 4), 150 nM PMA (lane 6) and a combination of 150 nM PMA and 100 nM okadaic acid (lane 8). Northern blot analysis was performed as described in the legend to Fig. 1, with a u-PAR cDNA probe (A) and a GAPDH probe (B).

is important for an understanding of the mechanism of the preferential expression of u-PAR in some cancer cells during invasion. The present study indicates that the signal transduction in many cells of neoplastic origin involves threonine/serine phosphorylation of one or more factors that induce u-PAR gene transcription. A further identification of these factors would be facilitated by the isolation and characterization of the u-PAR gene.

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