Int. J. Cancer: **75,** 744–749 (1998)

Publication of the International Union Against Cancer
Publication de l'Union Internationale Contre le Cancer

TWO-STAGE EXPOSURE OF SYRIAN-HAMSTER-EMBRYO CELLS TO ENVIRONMENTAL CARCINOGENS: SUPERINDUCTION OF ORNITHINE DECARBOXYLASE CORRELATES WITH INCREASE OF MORPHOLOGICAL-TRANSFORMATION FREQUENCY

Stéphane DHALLUIN[1], Zoé ELIAS[2], Véronique CRUCIANI[3], Halima BESSI[4], Odile POIROT[2], Claudine RAST[3], Laurent GATE[1], Nicole PAGES[1], Haim TAPIERO[1], Paule VASSEUR[3] and Giao NGUYEN-BA[1]*

[1]*Laboratory of Cellular and Molecular Pharmacology, CNRS-URA 1218, Faculty of Pharmacy, Chatenay-Malabry, France*
[2]*Laboratory of Carcinogenesis, INRS, Vandoeuvre les Nancy, France*
[3]*Environment Science Centre, Metz, France*
[4]*Faculty of Science and Technologies, Mohammedia, Morocco*

As part of environmental toxicology, it is important to assess both the carcinogenic potential of xenobiotics and their mode of action on target cells. Since dysregulation of ornithine decarboxylase (ODC), a rate-limiting enzyme of polyamine biosynthesis, is considered as an early and essential component in the process of multistage carcinogenesis, we have studied the mode of ODC induction in Syrian-hamster-embryo(SHE) cells stage-exposed to carcinogens and to non-carcinogens. One-stage (5 hr) treatment of SHE cells with 50 μM clofibrate (CLF), a non-genotoxic carcinogen, or with 0.4 μM benzo(a)pyrene (BaP), a genotoxic carcinogen, slightly decreased basal ODC activity. Using the 2-stage exposure, 1 hr to carcinogen, then replacement by TPA for 5 hr, the ODC activity was higher than that obtained with TPA alone. This ODC superinduction was not observed when SHE cells were similarly pre-treated with non-carcinogenic compounds. Several environmental chemicals, pesticides, solvents, oxidizers and drugs were investigated with this SHE cell model. With one-stage exposure, some xenobiotics decreased basal ODC activity, while for others ODC changes were not noticeable. With 2-stage exposure (chemical followed by TPA), all carcinogens amplified the TPA-inducing effect, resulting in ODC superinduction. Comparative studies of the action of carcinogens and of non-carcinogens, using 2-stage exposure protocols, clearly show a close relationship between ODC induction rate and morphological transformation frequency. *Int. J. Cancer* 75:744–749, 1998.
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Evaluation of the carcinogenicity of xenobiotics is considered to be an important aspect of environmental-toxicology studies. The multistage process of chemical carcinogenesis is controlled by a variety of factors, including genotoxic impacts as well as epigenetic events (Roe, 1989). Since current short-term tests to detect a chemical's carcinogenic potential typically evaluate the ability of a chemical to induce mutations or chromosome aberrations, chemicals that induce neoplasia by mechanisms other than these will not be detected (Yamasaki *et al.,* 1996). According to the results of the National Toxicology Bioassay Program (US NTP), approximately 20% of the chemicals found to be carcinogenic in rodents were negative in a standard genotoxicity test battery consisting of *Salmonella* mutation assays, mouse lymphoma assays and *in vitro* cytogenetic assays (Ashby and Tennant, 1991). New approaches are needed to detect carcinogens lacking the ability to initially induce genetic damage or inducing genetic changes not assessed in standard genotoxicity tests. Moreover, our understanding of the epigenetic mechanisms of carcinogenesis requires detailed knowledge of early changes in signal transduction, biochemical and molecular reactions, cell-cycle control and apoptosis in target cells. To meet this requirement, it is essential to design *in vitro* cell-transformation models that closely mimic the multistage process of carcinogenesis and to examine the mechanisms by which xenobiotic agents induce evaluable pre-neoplastic or neoplastic end-points. Two categories of cell transformation systems have been developed: in one, the established cell lines Balb/c 3T3 and C3H10T1/2 (aneuploid cells) are used in assays of transformed foci (Kakunaga and Yamasaki, 1985); in the second, early passages of diploid Syrian-hamster-embryo cells(SHE) are used in assays of transformed colonies. Several studies clearly showed that staged transformation of SHE cell closely mimics *in vivo* multistage carcinogenesis (Bessi *et al.,* 1995). SHE cells are representative of normal diploid cells, genetically stable, capable of activating a wide spectrum of chemical carcinogens and of undergoing apoptosis. The cells are sensitive to genotoxic and non-genotoxic carcinogens, forming transformed colonies as measurable end-points. The probability of progressing toward a neoplastic phenotype is greater for SHE cells derived from transformed colonies than for parental cells (Leboeuf *et al.,* 1996).

An important biochemical aspect of the epigenetic mechanism of carcinogenesis concerns the relationship between alterations of polyamine metabolism and cell progression toward neoplastic phenotypes. The first and rate-limiting enzyme of polyamine biosynthesis is ornithine decarboxylase (ODC, EC.4.1.1.17), whose activity is closely related to the cell-proliferation rate (Heby, 1981). In human cancers, as in chemically induced skin carcinogenesis in rodents, ODC was highly expressed (O'Brien, 1976; Clifford *et al.,* 1995). *In vitro,* the relationship between ODC over-expression and cellular neoplastic transformation was demonstrated with Nemalite-transformed SHE cells (Nguyen-Ba *et al.,* 1993), a transformed NIH/3T3 cell line and Rat-1 fibroblasts transfected with human ODC-cDNA in sense and anti-sense orientations (Auvinen *et al.,* 1992). Reduction of ODC activity in a dominant negative mutant of pMV7-4E cells reversed the transformed phenotype (Shantz *et al.,* 1996). Transgenic mice harboring activated human ODC-cDNA were more susceptible than wild-type mice to skin-tumor promotion by TPA (Halmekyto *et al.,* 1992). It has been reported that double transgenic mice v-ras^{Ha} TG.AC-K6/ODC, homozygous for v-*ras*^{Ha} mutated gene and over-expressing ODC, can develop skin tumors in the absence of any initiators and promoters (Gilmour *et al.,* 1997). In contrast, inhibition of ODC activity by α-difluoromethylornithine in mice fed with a low polyamine diet, was found to decrease tumor development (Verma *et al.,* 1996). This finding was applied in clinical trials aiming to improve chemotherapeutic treatments (Quemener *et al.,* 1992).

When SHE cells were exposed to environmental carcinogens, the ODC metabolism was altered. Studying these changes may help

Contract grant sponsor: French Ministry of the Environment; Contract grant number: Com. 36/95. Association for Research in Cancerology. 1380/96; Mitsuyoshi Nomura Foundation for Cancer and AIDS Research, Japan.

*Correspondence to: Laboratory of Cellular and Molecular Pharmacology, URA CNRS 1218, Faculty of Pharmacy, 5 JB Clement, 92290 Chatenay-Malabry, France. Fax: (33) 01.46.83.58.13. E-mail: Giao.Nguyen-Ba@cep.u-psud.fr

Received 15 July 1997; Revised 17 October 1997

to elucidate the development of early stages, probably critical to the carcinogenic process. In this work we have studied (i) the mode of ODC induction in 1-stage and 2-stage exposure of SHE cells to model carcinogens; (ii) the relationship between ODC superinduction and the cell-transforming potential of several environmental xenobiotics, pesticides, solvents, oxidizers and drugs. The ODC superinduction obtained in staged exposure of SHE cells is closely related to the carcinogenic potential of chemicals and to increased morphological transformation in SHE cells.

MATERIAL AND METHODS

Chemicals

In this study, we used genotoxic and non-genotoxic carcinogens, also, for comparison, some non-carcinogenic chemicals structurally closely related to carcinogens. Benzo(a)pyrene (BaP), 12-0-tetradecanoylphorbol-13-acetate (TPA), clofibrate (CLF), diethylhexylphthalate (DEHP), phthalic anhydride (AP), benzoyle peroxide (PBZ), 1,2-dichlorobenzene (oDCB), 1,4-dichlorobenzene (pDCB), 1-nitropopane (1NP), 2-nitropopane (2NP), ethylene glycol monomethyl ether (EGME), 4-O-methyl TPA (4a TPA), were purchased from Sigma-Aldrich (La Verpilière, France), 2-methoxyacetaldehyde (MALD) from INRS (Nancy, France) and chlordane (CLD) from Riedel-de-Haen (Seelze, Germany). Culture medium (DMEM) was purchased from GIBCO BRL (Eraguy, France); FCS from Hyclone (Gramlington, UK); L-(1-^{14}C) ornithine from Amersham (Aylesbury, UK); other chemicals were of RP grade.

Cell cultures and treatments

Primary cell cultures were prepared from 13-day-old golden-Syrian-hamster embryos, as described by Elias *et al.* (1989). In preliminary experiments, the first cell batch was tested for its capacity to develop transformed colonies in the presence of 4 µM of benzo(a)pyrene in a morphological transformation assay. Positive cell batches were stored in liquid nitrogen. Early diploid-cell passages are extensively used in SHE-cell morphological transformation assays and are a useful method for detecting the carcinogenic potential of chemicals. Its sensitivity is greatly increased by the adoption of a 2-stage protocol, initiation-promotion (Elias *et al.*, 1989; Bessi *et al.*, 1995). For the ODC activity assay, the cells are seeded into 12-well culture plates in DMEM medium, pH 7.4, supplemented with 15% FCS; 72 hr later they are submitted to chemical treatments as described (Dhalluin *et al.*, 1997). For each chemical, preliminary cytotoxic studies are undertaken. After treatment with various doses of xenobiotic, SHE cells are trypsinized, washed and stained with fluorescent dye solution: acridine orange (AO, 0.15 mg/ml) and ethidium bromide (BET, 0.5 mg/ml). Fluorescent microscopic (495 nm) observation reveals living cells permeable to AO, with green nuclei and dead cells, permeable to BET, with their nuclei stained orange. These orange-stained nuclei are homogeneous in necrotic cells, whereas condensed chromatin fragments are present in apoptotic cells. Doses of chemical inducing less than 5% necrotic cells after 5-hr treatment will be used in the next step of ODC assay. In the 1-stage-treatment protocol, SHE cells are exposed to non-toxic doses of xenobiotic; stimulation or inhibition of basal ODC activity may be observed after 5-hr treatment. In other experiments, SHE cells are exposed to an optimal dose of xenobiotic, and ODC activity is measured after 2-, 4-, 6- and 24-hr exposure. For several chemicals, 5 hr is the optimal exposure time. In the 2-stage-exposure protocols, SHE cells are submitted to the action of both xenobiotic and promoter phorbol-ester TPA: (i) to study the interaction of xenobiotic on TPA-induced ODC activity in the cells exposed to the concomitant effect of TPA and xenobiotic, this is applied at the same time as TPA or 1 hr before, or 2 hr after TPA treatment; (ii) in sequential exposure, cells are pre-exposed to xenobiotic for 1 hr (initiation stage), then to TPA for 5 hr (promotion stage); (iii) to study the effect of xenobiotic at the promotion stage, cells are pre-treated for 1 hr with 0.4 µM BaP, followed by 5-hr exposure to various doses of xenobiotic. Stage-exposure protocols used in this ODC study are closely related to stage-treatment methods used in morphological SHE-cell transformation assays.

Assay of ODC activity

After cell exposure to chemicals, cellular ODC activity was determined by measuring the $^{14}CO_2$ released from ^{14}C-labelled L-ornithine as described (Nguyen-Ba *et al.*, 1994). Briefly, lysed cell cultures were incubated at 37°C, in 50 mM Tris-HCl buffer, pH 7.5, 40 mM pyridoxal phosphate, 2.5 mM dithiothreitol, 0.4 mM L-ornithine containing 0.1 µCi (3.7 kBq) ^{14}C-labelled L-ornithine, 0.4 ml final volume. After 1-hr incubation, the enzymatic reaction was stopped by injecting 0.05 ml of 2N PCA. ^{14}C-labelled CO_2 released was adsorbed onto a filter disc (Whatman GF/C, Moutargis, France) and the radioactivity was determined by liquid-scintillation counting (Beckman counter LS-6000-TA, Palo Alto, CA). Protein concentration was measured according to the Bradford method on a Labsystems (Les Ulis, France) Multiskan MS microplate reader. Statistical analyses were performed using Student's *t*-test. ODC activity was primarily expressed as nmol $^{14}CO_2$/mg protein/60 mn.

RESULTS

Preliminary studies have been done with xenobiotics to determine optimal doses without cytotoxic effect, as well as the optimal exposure time of SHE cells to chemicals. Usually 3 doses of xenobiotic were applied to cell cultures (in triplicate). In view of the results, one dose was selected for other timed treatments: 2, 4, 6 and 24 hr. In cells exposed to 25, 50 and 100 µM CLF, the decrease of ODC activity was approximately 30, 40 and 70%. SHE cells were then exposed to 50 µM CLF; 30% of ODC inhibition was seen after 2 hr and 40% inhibition after 4- and 6-hr exposure. For various chemicals, 5 hr was found to be the optimal exposure time.

Two-stage exposure of SHE cells to genotoxic carcinogen BaP and to TPA

Treatment for 5 hr with 0.16 µM TPA increased the ODC activity from 100 to about 270%, whereas 0.40 µM BaP decreased this activity by about 20% below basal level (Fig. 1). In concomitant exposures of SHE cells to TPA and BaP, this compound was applied together with TPA for 5 hr, or 1 hr before TPA or 2 hr after TPA. In these assays, BaP did not significantly modulate the effect of TPA on ODC activity. Surprisingly, in 2-stage exposure, 1 hr to BaP followed by 5 hr treatment by TPA, the ODC activity strongly increased, to 325%, higher than that obtained with TPA alone.

Effect of the non-genotoxic carcinogen CLF and TPA on ODC regulation

After 5-hr exposure of SHE cells to 50 µM of hypolipidemic drug CLF, the ODC activity decreased from 100 ± 8% to 60 ± 2%. After 5 hr in the presence of 0.16 µM TPA, the ODC activity increased to 196 ± 20%. Following 2-stage exposure, 1 hr to 50 µM CLF (initiation), then 5 hr in the presence of 0.16 µM TPA (promotion), the ODC activity strongly increased, to a level far higher than that obtained with TPA alone, from 196 to 272% respectively (Fig. 2). When the cells were co-treated with 50 µM CLF and 0.16 µM TPA for 5 hr the ODC activity was reduced to 130%, below the level obtained with TPA alone. To explore the effect of CLF at the promotion stage, cells were pre-treated with BaP (0.40 µM) followed by 5-hr exposure to 50 µM CLF. As compared with the basal level, the ODC activity decreased by 24% when cells were treated with BaP alone and decreased by 40% when cells were sequentially treated with BaP and CLF.

In the course of this study, the effect of several carcinogens and non-carcinogens on ODC activity was investigated, using 1-stage and 2-stage SHE-cell exposure. In 1-stage treatment, 5 hr, only 0.16 µM TPA strongly induced ODC activity. Optimal doses of other chemicals could not significantly increase ODC activity. Some of them decreased this basal ODC activity, particularly 0.16 µM 4a-TPA, 50 µM CLF, 20 µM PBZ, 10 mM 1-NP, 10 mM 2-NP and 200 µM MALD. With other chemicals, ODC changes were not

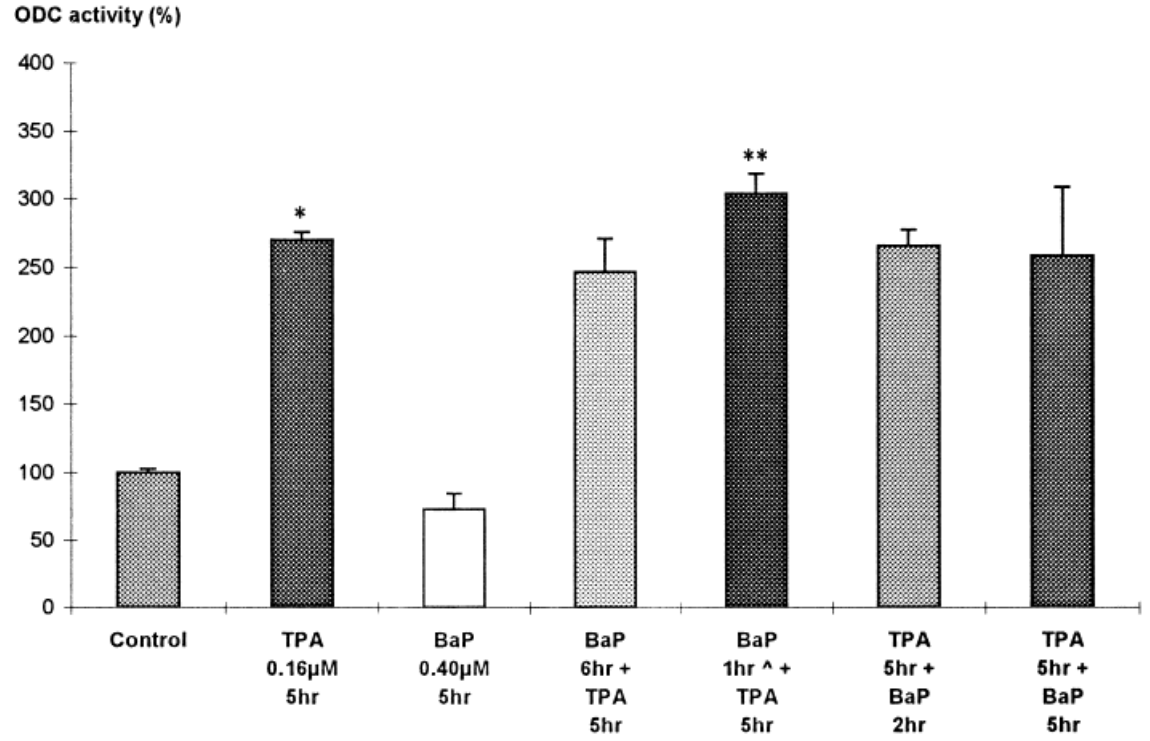

FIGURE 1 – Superinduction of ODC activity in stage-exposed SHE cells to benzo[a]pyrene (BaP) and phorbol ester TPA. Exposures were performed in DMEM, pH 6.7, supplemented with 5% FCS. BaP 1 hr + TPA 5 hr consisted of 1-hr exposure to 0.40 µM BaP then removed and replaced for 5 hr by 0.16 µM phorbol ester TPA. Each bar represents the mean of triplicate determinations; 2 further independent experiments yielded similar results. *ODC activity significantly different from control ($p < 0.05$); **ODC activity significantly different from that obtained after TPA exposure ($p < 0.05$).

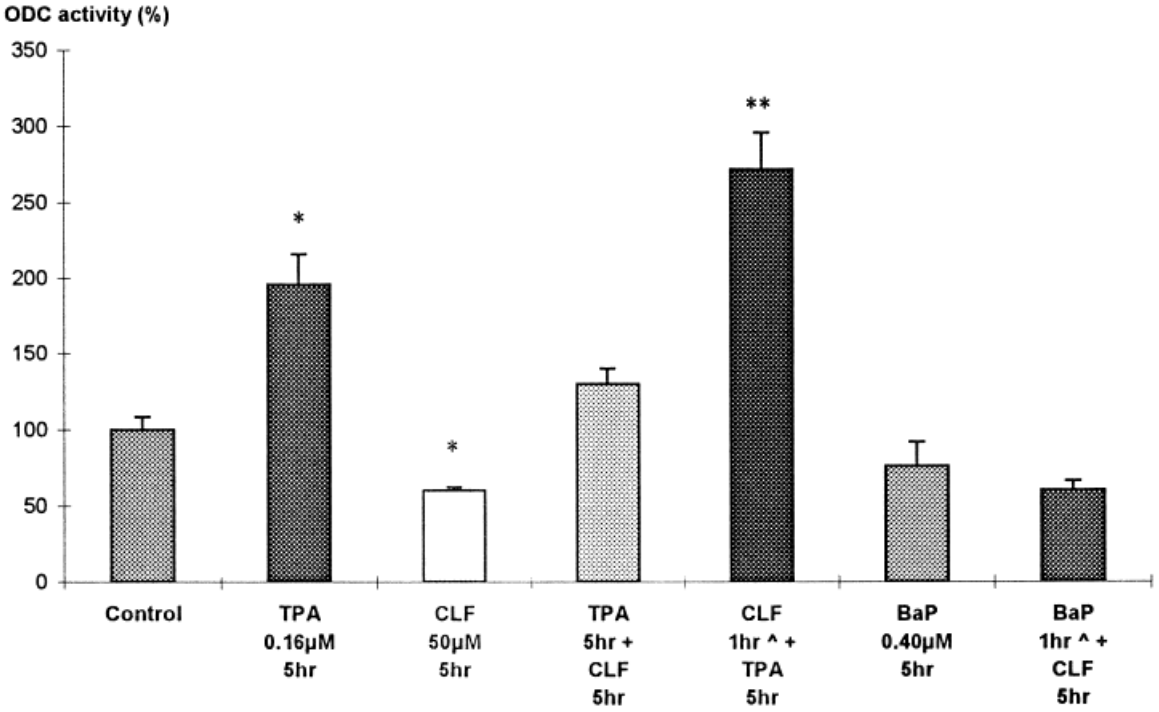

FIGURE 2 – Modulation of ODC activity in SHE cells exposed to clofibrate (CLF) in initiation and in promotion stage. Exposures were performed in DMEM, pH 6.7, supplemented with 5% FCS. CLF 1 h + TPA 5 hr consisted of 1-hr exposure to 50 µM CLF, which was then replaced by 0.16 µM phorbol ester TPA for 5 hr. BaP 1 hr + CLF 5 hr consisted of 1-hr exposure to 0.40 µM BaP, which was then replaced for 5 hr by 50 µM CLF. Each bar represents the mean of triplicate determinations; 2 further independent experiments yielded similar results. *ODC activity significantly different from control ($p < 0.05$); **ODC activity significantly different from that obtained after TPA exposure ($p < 0.05$).

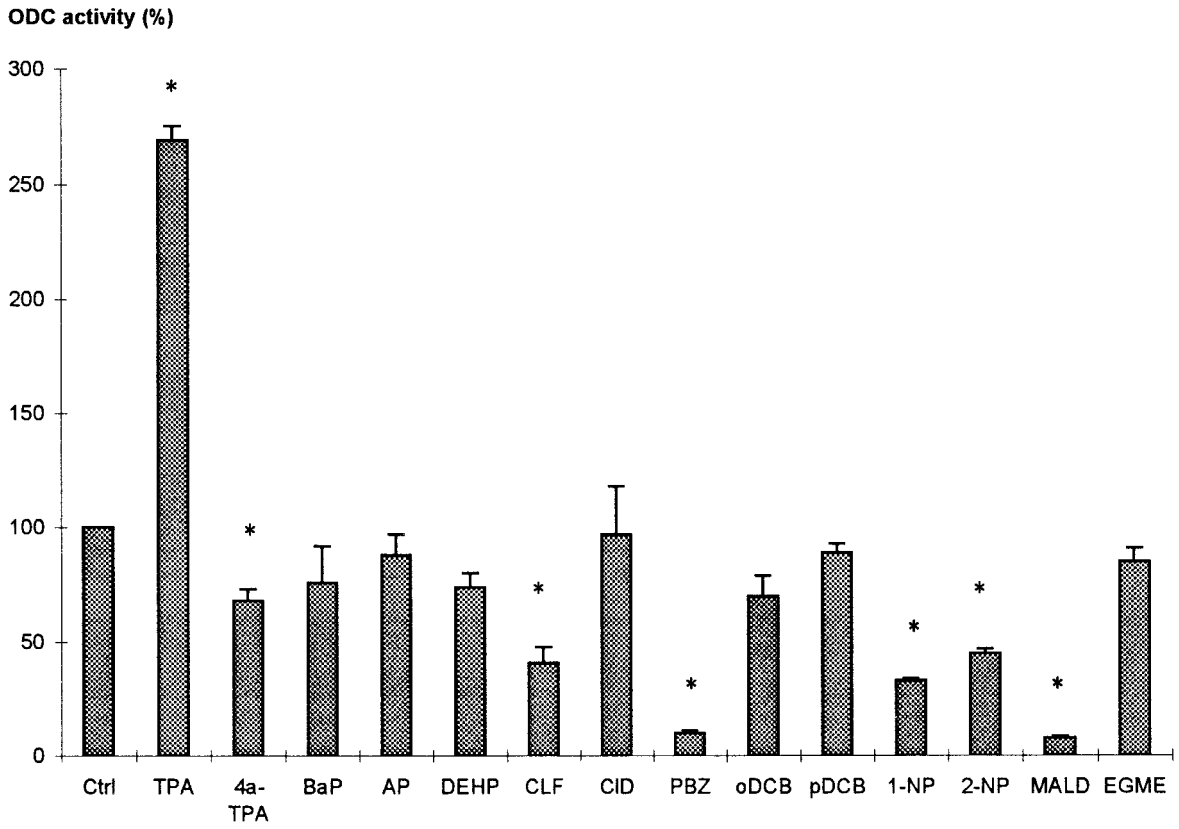

FIGURE 3 – Modification of ODC activity after 1-stage exposure of SHE cells to environmental carcinogens and non-carcinogens. One-stage exposure consisted of 5-hr exposure to carcinogens or non-carcinogens. Chemical doses are the same as reported in Table I. *ODC activity significantly different from control Ctrl ($p < 0.05$).

noticeable (Fig. 3). The same pattern of cell responses was observed with a 24-hr exposure time.

Surprisingly, in 2-stage exposure (Fig. 4), 1 hr to carcinogen followed by 5-hr treatment with 0.16 μM TPA, all carcinogenic chemicals were able to stimulate ODC activity above the level induced by TPA alone. That is the case for genotoxic and non-genotoxic carcinogens BaP, DEHP, CLF, ClD, PBZ, pDCB, 2-NP. This superinduction was not observed when non-carcinogenic derivatives (4a-TPA, AP and oDCB) were used in the initiation stage. No published data are available about the *in vivo* carcinogenic potential of EGME and its metabolite MALD. ODC superinduction was obtained with MALD, a mutagenic agent, while the parent compound EGME, negative in the Ames test, did not enhance the ODC induction rate.

Table I recapitulates results of morphological transformation induced by chemicals in SHE cells, and compares them with ODC changes reported in this study. It can be seen that use of the 2-stage treatment of SHE cells by xenobiotics, to compare morphological transformation and ODC induction, clearly showed a relationship between the 2 assays: ODC superinduction and increase of cell-transformation frequency were obtained only with carcinogenic compounds. The rate of concordant responses between ODC induction and morphological transformation was 7 to 7 with carcinogens, while negative biochemical and biological responses were observed with 4 non-carcinogenic chemicals. One exception was ODC superinduction and increased transformation frequency with 10 mM 1-NP, a chemical classified as non-carcinogenic in rodent assays.

DISCUSSION

Superinduction of ODC activity in SHE cells stage-exposed to carcinogens could be an early and critical event in the SHE-cell transformation process, which, *in vitro* mimics chemically induced carcinogenesis in rodents. Using a 1-stage treatment protocol, Leboeuf *et al.* (1996) reported that, with more than 500 chemical, physical and biological agents tested in the SHE-cell assay, 80 to 90% concordance was obtained between morphological transformation and carcinogenicity. In order to increase the assay sensitivity, particularly with weak carcinogens, the 2-stage treatment protocol could be used. Results obtained with ODC studies strengthen this approach of sequential treatment (carcinogen followed by TPA). ODC superinduction following 2-stage exposure may reflect an increasing rate of cell proliferation, particularly of some initiated or mutated cells. A high level of cellular ODC may also intervene in the process of apoptosis (programmed cell death). Some carcinogens are shown to decrease the apoptotic rate in SHE cells (Dhalluin *et al.*, 1997), which could hamper the elimination of abnormal cells. Inhibition of apoptosis is related to activation of *Bcl*-2 proto-oncogene expression; a high level of Bcl-2 oncoprotein has been detected in various human cancers, such as colorectal, gastric and prostatic tumours, also in neuroblastoma, melanoma, leukemia and thyroid cancer. Since perturbation of the equilibrium between cell proliferation and cell death is considered as an essential event in the process of carcinogenesis (Schulte-Hermann *et al.*, 1995), it is important to establish some correlation between 2-stage exposure of SHE cells to carcinogens and the inhibition of apoptosis in this cell system.

The mechanism of ODC superinduction after stage exposure of SHE cells to carcinogens is not clearly elucidated. ODC is regulated at multiple levels, from ODC gene expression to protein catabolism. Western-blot analysis gives concordant results between the increase of ODC protein synthesis and a high rate of ODC activity. The discrepancy appears in relation to ODC mRNA expression; where the induction rate is no different for 2-stage exposure to carcinogens and for 1-stage exposure to TPA alone.

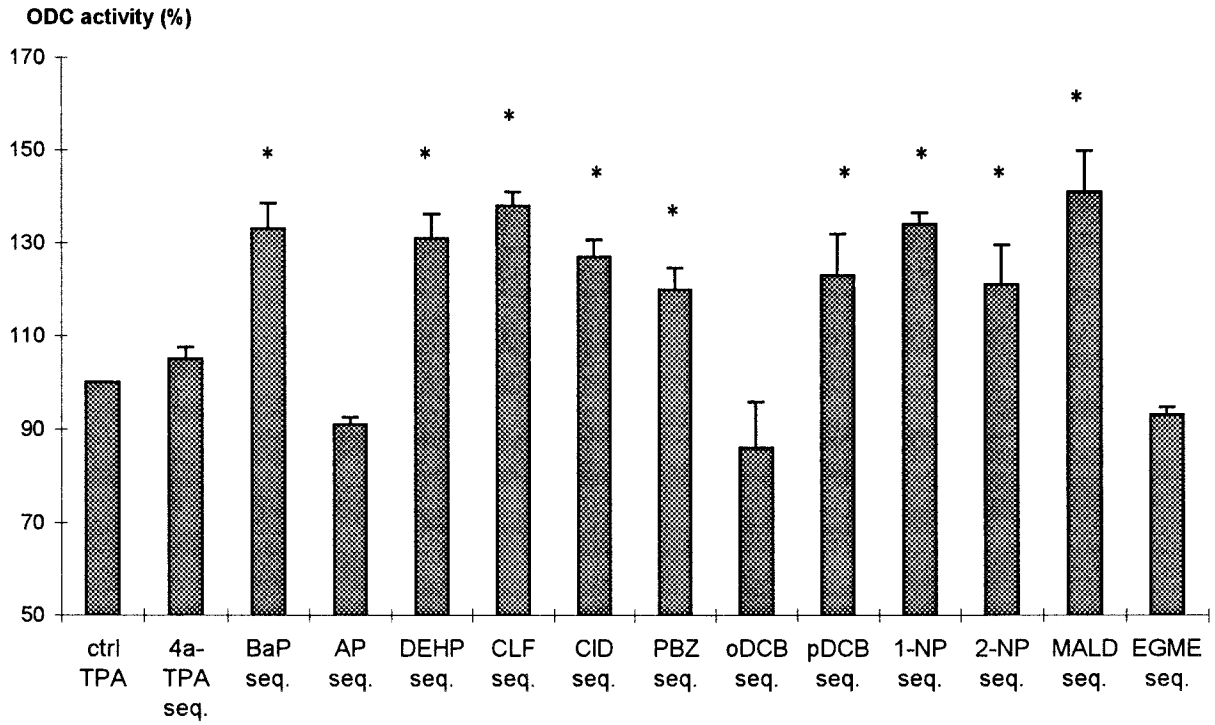

FIGURE 4 – Superinduction of ODC activity after 2-stage exposure of SHE cells to environmental carcinogens and phorbol-ester TPA. Two-stage exposure (xenobiotic seq.) consisted of 1-hr exposure to carcinogen or non-carcinogen, after which xenobiotic was replaced by phorbol-ester TPA for 5 hr. Chemical doses are the same as reported in Table I. Results are expressed as percentage of ODC activity compared with TPA exposure used as a control and set at 100% (ctrl TPA). Results represent the mean of triplicate determinations; 2 further experiments yielded similar results. *ODC activity significantly different from that obtained after 5-hr TPA exposure ($p < 0.05$).

TABLE I – RECAPITULATIVE TABLE COMPARING ODC ACTIVITY AND SHE-CELL MORPHOLOGICAL-TRANSFORMATION DATA FOLLOWING 1-STAGE AND 2-STAGE EXPOSURES TO ENVIRONMENTAL CARCINOGENS

Chemicals	ODC activity		Morphological SHE-cell transformation		*In vivo* carcinogenesis[1] rodent assays	*Salmonella* assay response
	One-stage treatment (chemical alone) ODC induction	Two-stage treatment (X1-hr + TPA5 hr) ODC superinduction	One-stage 7-day exposure (chemical alone)	Two-stage 7-day exposure (chemical/TPA) increased rate		
Benzo[a]pyrene 0.4 µM[2]	0	+	+	+	+	+
TPA 0.16 µM	↗	(+)	+	n.t.	+	−
Chlordane 20 µM[3]	0	+	+	+	+	−
Clofibrate 50 µM[4]	↘	+	+	+	+	−
Diethylhexylphthalate 100 µM[5]	↘	+	+	+	+	−
1,4-dichlorobenzene 70 µM[6]	0	+	+	n.t.	+	−
2-methoxyacetaldehyde 200 µM[7]	↘	+	+	+	n.t.	+
2-nitropropane 10 mM[6]	↘	+	−	+	+	+
Benzoyle peroxide 20 µM[6]	↘	+	+	+	+	−
Phthalic anhydride 135 µM[6]	0	−	−	−	−	−
1,2-dichlorobenzene 70 µM[6]	↘	−	−	n.t.	−	−
Ethylene glycol monomethyl ether 2 mM[7]	0	−	−	−	n.t.	−
4-O-methyl TPA 0.16 µM[6]	0	−	−	−	−	−
1-nitropropane 10 mM[6]	↘	+	−	+	−	−

[1]ODC activity data: 1-stage and 2-stage exposures were performed in DMEM, pH 6.7, supplemented with 5% FCS. One-stage exposure consisted of 5-hr exposure to xenobiotic; 2-stage exposure (X 1 hr + TPA 5 hr) consisted of 1-hr exposure to xenobiotic followed by 5-hr to phorbol-ester TPA. 0, no modification of ODC activity; ↘, inhibition of basal ODC activity; ↗, stimulation of basal ODC activity; +, over-induction of ODC activity; −, no over-inducing effect. Morphological transformation data: +, increase of morphological transformation frequency; −, no effect; n.t., not tested.–[2]Cruciani *et al.* (1996).–[3]Bessi *et al.* (1995).–[4]Dhalluin *et al.* (1996).–[5]data not shown.–[6]data not shown.–[7]Hoflack *et al.* (1995). Ashby and Tennant (1991). Data concerning carcinogenicity of glycol ethers are not available; MALD is a mutagenic compound, EGME is not.

Several sites of ODC regulation may be involved: intervention of the 5′ untranslated region (5′UTR), ODC-protein phosphorylation and dephosphorylation turnover, and ODC catabolism. Particularities in ODC catabolism concern the interaction of ODC antizyme, which, complexing with ODC, could (i) inactivate the enzyme, (ii) trigger proteolytic machinery through 26S proteasome, probably through a soluble 72-kDa protease identified in SHE cells (Nguyen-Ba *et al.*, 1994). Another pathway that increases TPA action may be related to change in protein-kinase-C activity. However, *in vivo* exploration in mouse epidermal cells gives no correlation between ODC superinduction and the down-regulation rate for PKC isoenzymes (Verma *et al.*, 1996).

Screening studies using environmental carcinogens show that, except in the case of TPA, ODC activity is not induced by 1-stage

treatment. Probably in a short interval of time, all changes induced in ODC metabolism by carcinogen could immediately be under repair. Our measurement methods are not sensitive enough to detect these kinetic disturbances in ODC metabolism. In the 2-stage treatment protocol, specific changes in ODC expression by carcinogen may be amplified by TPA action in the second stage, the result being ODC superinduction. Within the same SHE-cell system, the comparison of morphological transformation rate with ODC level shows a close relationship between the 2 assays: the concordance rate is 100% when 2-stage exposure protocols are used. The comparative list needs to be enriched by new data from carcinogenic chemicals and mineral fibers now under investigation.

Since the constitutive level of ODC is elevated in tumoral cells, our research programme may help to establish ODC superinduction as a biochemical marker of an early stage in the cell-transformation process. Biological and biochemical studies with this SHE-cell system may clarify the mode of action of carcinogens, their carcinogenic potency, the interaction of multiple exposures and the effect of concomitant or sequential exposure to environmental agents.

ACKNOWLEDGEMENTS

We are indebted to Dr L.L. Pritchard (Villejuif, France) for helpful discussion and for a careful review of the manuscript.

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