ALTERATIONS OF SIGNAL TRANSDUCTION PATHWAYS INVOLVED IN 2,3,7,8-TETRA CHLORO DIBENZO-P-DIOXIN-INDUCED MALIGNANT TRANSFORMATION OF HUMAN CELLS IN CULTURE

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ABSTRACT
Effects of signal transduction pathways in TCDD-induced neoplastic transformation of human cells were assessed with respect to PLC-coupled signaling pathways, adenylyl cyclase-mediated responses and PKC isozyme expressions. A lower stimulation of the intracellular free calcium levels with exposure to extracellular ATP or histamine was observed in the transformed cells, as compared to the parental cells. While the steady-state level of IP$_3$ was higher in the transformed cells, the magnitude of stimulation of IP$_3$ generation by ATP or histamine was significantly lower in the transformed cells than the parental cells. These results indicate that a downregulation of PLC-coupled signaling pathways may be involved in the TCDD-induced transformation of human cells. While the steady-state levels of cAMP accumulation were similar between the two cell lines, treatment of PGE$_2$, a potent differentiation inducer, stimulated a higher accumulation of cAMP in the parental cells but isoproterenol, a typical β-adrenergic agonist, did not induce a significant difference between the two cell lines. These results suggest that desensitization of cAMP-mediated responses to extracellular signals including differentiation signals may be
associated with a possible mechanism of the carcinogenesis. Elevated expression of PKC-α, -γ, -ζ, -ε, -δ, and -λ were observed in TCDD-transformed cells, indicating a possible association of altered expression of PKC isozymes with TCDD-induced transformation of human cells. The present study demonstrates that alterations of signal transduction pathways are involved in the TCDD-induced transformation of human cells and provides a valuable basis to investigate effects of signaling pathway as a possible mechanism of TCDD-induced carcinogenesis in human cells.

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INTRODUCTION

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is one of the most potent carcinogens ever tested in animal assays and may be known as a human carcinogen at least at high doses [1]. However, studies on the mechanism of TCDD-induced carcinogenesis in humans have been limited due to the lack of adequate human cellular models. Recently, Yang et al. reported TCDD-induced neoplastic transformation of an immortalized human keratinocyte cell line, which showed increases in colony formation in soft agar and cellular density in confluent culture and tumorigenicity in the nude mouse assay [2]. Since the cellular model used in their previous study is the only human cell line ever reported to show a malignant transformation by TCDD and it is of epithelial origin, a malignant transformation of this cellular system may provide an opportunity to look at various aspects, including signal transduction pathways, of potential mechanisms of TCDD-induced carcinogenesis in human cells.

Phosphoinositides are ubiquitous components of eukaryotic cell membranes and play crucial roles in signal transduction pathways [3]. Activation of phosphoinositide metabolism following receptor-agonist binding leads to phospholipase C-dependent hydrolysis of PIP₂, which generates two second messengers; IP₃ and DAG. Alteration of these second messengers has been associated with cell proliferation and malignancy. Increased production of IP₃ with malignant transformation has been observed in several cellular models. In particular, alterations of inositol phospholipid signaling pathways have been frequently reported in oncogene-induced malignant transformation [3,4].

cAMP plays an important role in changes of PLC activities or phosphoinositide kinase activity and is associated with altered responses of tumor cells to extracellular
signals. Decrease of cAMP induction by differentiation inducers was observed in the H-ras-induced transformation of both canine and human cells [4,5]. Intracellular cAMP level was reported to regulate phenotypic characteristics of the H-ras transformed NIH3T3 cells and phospholipid metabolism in the H-ras transformed canine cells [6].

PKC triggers various cellular events associated with tumor promotion, enhanced expression of oncogenes and cell proliferation [7]. In particular, altered expressions of individual PKC isozymes are now known to play pivotal roles in mediating cellular transformation [8]. Overexpression of PKC-δ and -e showed opposite effects on malignant transformation of NIH3T3 cells [9]. Transformation of R6 cells by c-ras or v-fos led to an increased expression of PKC-α and -δ and a decreased expression of PKC-e [10].

While information on signal transduction pathway associated with cellular transformation is plentiful and various effects of TCDD on cellular events are reported in several studies [11,12], few studies have assessed effects of signal transduction pathways on TCDD-induced transformation of human cells. Thus, the present study has attempted to examine association between altered signaling pathways and TCDD-induced transformation.

In the present communication, it is demonstrated that, while constitutive elevation of IP3 level was observed in TCDD-transformed cells, induction of IP3 production and intracellular free calcium concentration by extracellular signal was significantly lower in the transformed cells than in the parental cells. Stimulation of cAMP accumulation by a differentiation inducer was also lower in the transformed cells. Overexpression of various PKC isozymes was detected in the transformed cells, as compared to the parental cells. These results indicate that alterations of signaling pathways are closely associated with TCDD-induced carcinogenesis in human cells.
MATERIALS AND METHODS

Cell Cultures and Media. The human epidermal keratinocyte line, designated RHEK-1, and its TCDD-transformed cell line were used for the present study. The parental cell line (RHEK-1) was established from primary foreskin epidermal keratinocytes after infection with the Ad12-SV40 hybrid virus [13]. This cell line had a 'flat' epithelial morphology, expressed a number of epithelial markers and was non-tumorigenic in nude mice. The TCDD-transformed cell line was established from RHEK-1 cells after 2 week exposure of 0.3 nM TCDD and 6 subcultures, as previously reported [2]. This cell line showed a variety of cellular transformation properties and tumorigenicity in nude mice assay. Growth and maintenance medium for these cell lines consisted of Dulbecco's modified minimal essential medium (GIBCO, Gaithersburg, MD) with 10% fetal bovine serum, hydrocortison (5 μg/ml), penicillin G (50 u/ml), and streptomycin (50 μg/ml).

Measurement of Intracellular Free Calcium Level. Intracellular free Ca\(^{2+}\) concentration was determined using the fluorescent Ca\(^{2+}\) indicator fura-2 as previously reported [14]. Briefly, parental cells or TCDD-transformed cells were loaded with fura-2 acetoxymethylester (fura-2/AM) (Molecular Probes, Eugene, OR) to a final concentration of 3 μM in the complete medium and incubated at 37 °C with stirring for 50 min. Fluorescence ratios were taken by dual excitation at 340 nm and emission at 500 nm using an alternative wavelength time scanning method. Calibration of the fluorescence signal in terms of intracellular free calcium concentration was performed according to Grynkiewicz et al [15]. In the extracellular Ca\(^{2+}\)-free experiments, Locke's solution contained 200 μM EGTA instead of Ca\(^{2+}\)-ion.

Measurement of IP\(_3\) Level. IP\(_3\) concentrations in the cells were determined by competition assay with \(^{3}H\)IP\(_3\) (NEN, Boston, MA) in binding to IP\(_3\) binding protein as previously described [14]. Cells were grown in six well culture plates up to 90% of the confluency and treated with 100 μM ATP or 300 μM histamine for 20 sec. The reaction was terminated by aspirating off the medium and adding 0.3 ml of ice-cold 15% (wt/vol) TCA with 10 mM EGTA. The plates were left on ice for 30 min to extract the water-soluble inositol phosphates. A 20 μl aliquot of the cell extract was
added to 20 μl of the assay buffer (0.1 M Tris buffer containing 4 mM EDTA and 4 mg/ml bovine serum albumin) and 20 μl [3H]IP3 (0.1 Ci/ml). A 20 μl aliquot of solution containing the binding protein was added and the mixture was incubated for 15 min on ice and centrifuged at 2,000g for 10 min. A 100 μl aliquot of water and 1 ml of scintillation cocktail were added to the pellet to measure radioactivity. IP3 concentration in the sample was determined based on a standard curve and expressed as picomoles per microgram of protein. IP3 binding protein was prepared from bovine adrenal cortex according to the method of Challiss et al. [16].

**Measurement of [3H]cAMP Formation.** Intracellular cAMP was determined by measuring the formation of [3H]cAMP from [3H]adenine nucleotide pools as described previously by Salomon with some modifications [17]. The cells were grown in six-well plates to confluence and loaded with [3H]adenine (2 μCi/ml) (NEN, Boston, MA) in complete medium for 24 h. Cells were then washed three times with Locke’s solution and preincubated with Locke’s solution containing 1 mM IBMX (Research Biomedicals, Natick, MA) for 15 min. Stimulating buffer also contained IBMX. The reaction was stopped by adding 5% TCA containing 1 μM cold cAMP and the plates were left on ice for 30 min to extract water-soluble cAMP. Extracts were then centrifuged at 5,000g for 5 min to precipitate cell debris. [3H]cAMP and [3H]ATP were separated by sequential chromatography on Dowex AG50W-X4 (200-400 mesh) cation exchanger and neutral alumina column. [3H]ATP fraction was obtained by eluting Dowex column with 2 ml of distilled water and the subsequent eluents of the Dowex column with 3.5 ml of water was loaded to the alumina column. The alumina column was then washed with 4 ml of 0.1 M imidazole solution (pH 7.2) and the eluants were collected into scintillation vials containing 15 ml of scintillation fluid to determine the [3H]cAMP. Increases of intracellular cAMP concentration were calculated as [3H]cAMP/(μM [3H]ATP + [3H]cAMP) x 10^3.

**Western Blot Analysis of Protein Kinase C Isozymes.** Cells at 70% confluence were lysed in lysis buffer (50 mM HEPES, 150 mM NaCl, 2.5 mM EGTA, 1 mM EDTA, 1 mM DTT, 1% NP-40, 10% glycerol, 1 mM PMSF, 10 μg/ml aprotinin and 10 μg/ml leupeptin, adjusted to pH 7.5), then sonicated twice for 10 s on ice. Proteins (25
μg) of the lysates from parental cells or TCDD-transformed cells were separated on 8% SDS-PAGE and transferred to nitrocellulose membrane. The nitrocellulose sheet was blocked with 3% non-fat dry milk in Tris buffered saline. PKC isozymes such as α, β, γ, ε, θ, λ, and ε were detected with isozyme-specific anti-PKC monoclonal antibodies (Transduction Laboratories, Lexington, KY), while δ and ζ isozymes were detected with polyclonal antibodies (GIBCOBRL, Gaithersburg, MD). The PKC antibodies bound to the proteins on the nitrocellulose sheets were detected with goat anti-rabbit or anti-mouse IgG conjugated with peroxidase using the ECL system of Amersham Life Sciences (Arlington Heights, IL).

Results

**Alterations of PLC-coupled Signal Transduction Pathway in TCDD-transformed Cells.**

When cells were stimulated with 100 μM extracellular ATP in presence or absence of extracellular calcium, parental cells showed significantly higher increases of the intracellular free calcium levels from the basal level than TCDD-transformed cells. (Fig. 1, A and B). While ATP treatment in presence of 2.2 mM extracellular calcium evoked additional induction of the intracellular free calcium levels in both cell lines, the magnitude of the increases from the respective basal level was 2.4 fold higher in parental cells than in transformed cells. Stimulation of cells with 300 μM histamine in presence or absence of extracellular calcium also resulted in a significantly higher induction of the intracellular free calcium level in parental cells than in transformed cells (Fig. 1, C and D). Addition of 2.2 mM calcium to the media induced additional increases of the intracellular calcium concentration in both parental and TCDD-transformed cells. However, the magnitude of the increases from the respective basal level by extracellular calcium was 1.9 times higher in the parental cells. These results suggest that a lowered function of Ca²⁺ mobilization from the intracellular calcium stores and the extracellular space be associated with TCDD-induced carcinogenesis of this particular human cell system. When steady-state IP₃ levels were measured, TCDD-transformed cells showed a higher concentration than parental cells. However, when cells were stimulated with 100 μM extracellular ATP or
300 μM histamine, levels of IP₃ induction was significantly lower in the transformed cells than in the parental cells (Fig. 2).

**Responses of Adenylyl Cyclase-mediated Signals in Parental and TCDD-transformed Cells.**

Steady-state levels of cAMP were similar between parental and transformed cells. To evaluate responses of adenylyl cyclase-mediated signaling pathway, cells were treated with various types of cAMP-inducing agonists. Forskolin-treated transformed cells exhibited less cAMP accumulation than parental cells. Treatment of cells with PGE₂ also resulted in a lower level of induction of cAMP accumulation in the transformed cells. However, no significant differences in cAMP accumulation between the two cell lines were observed upon treatment with isoproterenol (Fig. 3).

**Elevated Expressions of PKC Isozymes in TCDD-transformed Cells.**

The expression of different PKC isozymes in parental cells and TCDD-transformed cells was determined by western blotting with isozyme-specific antibodies. All of the antibodies recognized all immunoreactive bands from the lysate of mouse brain as a positive control. Antibodies against PKC -α, -γ, -ε, -ζ, -λ, or -η isozymes detected higher levels of immunoreactive proteins from the lysates of TCDD-transformed cells, as compared to that of parental cells (Fig. 4). This is a first demonstration that these isozymes are expressed in this particular cellular system and their elevated expressions is associated with TCDD-induced transformation of human cells. PKC -β, -δ and -θ, however, do not seem to be expressed to a significant level as the corresponding antibodies failed to detect immunoproteins from either parental or transformed cell lysates.

**DISCUSSION**

We have evaluated effects of second messenger molecules associated with malignancy. Increase of IP₃ production has been reported in a variety of transformed cells such as H-ras transformed MDCK cells [18], rat embryo fibroblasts [19] and v-src transformed rat-1 fibroblasts [20]. NIH3T3 cells transformed by chemicals such as 3-MC have also shown increased production of IP₃ [21]. Consistent with these reports,
Fig. 1. Effects of ATP or histamine on increases of [Ca²⁺]ᵢ in parental cells and TCDD-transformed cells. The Ca²⁺ profiles from a typical experiment (top); pattern of [Ca²⁺]ᵢ increases in parental cells (A and C) and TCDD-transformed cells (B and D) in presence (solid line) or absence (dashed line) of 2.2 mM extracellular Ca²⁺. Increases of [Ca²⁺]ᵢ stimulated by ATP or histamine (bottom); four independent experiments were performed and the means of the representative triplicate determinations were presented. *, P < 0.05, as compared to parental cells.
Fig. 2. Effects of ATP or histamine on IP₃ generation in parental cells (open bar) and TCDD-transformed cells (solid bar). *, p < 0.05 as compared to parental cells.
our results showed constitutive increase of IP$_3$ production in TCDD-transformed cells, as compared to parental cells. The present study provides a further evidence that elevation of IP$_3$ production is associated with cellular transformation.

Extracellular ATP and histamine are known to be important signal molecules in various tissues which stimulate phospholipase C and increase IP$_3$ production. They also stimulate a rise in cytosolic free calcium concentration by internal calcium mobilization and calcium influx from the extracellular space [22]. When parental cells and TCDD-transformed cells were treated with these signal molecules, the level of IP$_3$ increase was significantly lower in the transformed cells than in the parental cells. In addition, levels of the intracellular free calcium concentration spikes by these agents were also lower in the transformed cells than in the parental cells, in absence or presence of extracellular calcium, suggesting an altered function of Ca$^{2+}$ mobilization mechanisms including calcium ion channels in TCDD-transformed cells. These results indicate that down-regulation of PLC-coupled signal transduction pathway may be, in part, involved in TCDD-induced malignancy.

While elevated production of IP$_3$ was generally observed in the transformed cells, decreased or steady-state levels of intracellular cAMP were reported in the H-ras transformed cells [23]. Consistent with these findings, we also observed that steady-state level of intracellular cAMP in TCDD-transformed cells was similar to that of parental cells. However, when cells were treated with cAMP inducers, differential responses between the two cell lines were observed. cAMP accumulation in forskolin-treated transformed cells was significantly lower than in parental cells. This is in contrast with a previous report demonstrating an equivalent stimulation of cAMP accumulation by forskolin treatment in both human immortalized cell line (RHEK-1), which is the same parental cell line used in the present study, and its ras-transformed cell line [5]. Thus, it is suggested that cellular transformation by TCDD may be involved in an alteration of signaling pathway different from the oncogene-derived cellular transformation. Both isoproterenol and PGE$_2$ are cAMP inducers, but PGE$_2$ is known to exert a strong cell differentiation induction via cAMP-dependent pathway. While isoproterenol treatment provided a similar stimulation of cAMP accumulation in both cell lines, PGE$_2$ treatment showed a significant reduction of cAMP accumulation in the TCDD-transformed cells. This observation suggests that lack of response to differentiation signals play a role in TCDD-induced carcinogenesis of the present
human cell system. In addition, since dysfunction of PGE$_2$ receptor is related to
tumorigenic phenotype of the cell and loss of PGE$_2$ control is associated with tumor
progression [24], further studies are warranted to determine if a possible defect in
PGE$_2$ receptor may be associated with the altered signaling pathway. Characterization of
PGE$_2$ receptor in the transformed cells will be required to clarify the evidence of
receptor alteration.

Considerable evidence now indicates that differential regulation of PKC
isozymes are closely implicated with malignant transformations [25]. Expression of
PKC-α is reported to be associated with human melanoma and astrocytoma [4,25].
Overexpression of PKC-ζ was observed in B- and T-lymphocytic neoplasm [25].
Altered expression of PKC-α, -β, -ε, and -ζ were also reported in the H-ras-
transformed R 6 rat embryo fibroblasts [10]. In particular, a regulation of PKC-ε
associated with malignant cell transformation has been in conflict findings. Mischak et
al. reported that overexpression of PKC-ε in NIH3T3 cells induced increases in cell
growth in soft agar and cell density in confluent culture and tumorigenicity in nude
mice [9]. In contrary, Kahl-Ranier et al. observed decreased expression of PKC -α, -ζ,
and -ε in malignant human colonic epithelial cells [26]. Boner et al. reported increased
expression of PKC-α and a concomitant decrease of PKC-ε in the H-ras-transformed
rat embryo fibroblasts and liver epithelial cell lines [27]. While alterations of PKC
isozyme expressions in the transformed cells are different by cell types, species or
transforming agents, TCDD-induced transformation of human cells in our study
revealed overexpression of PKC-α, -γ, -ε, -λ, -ζ, and -τ in TCDD-transformed cells.
This is a first evidence to indicate a possible association between overexpression of
these PKC isozymes and TCDD-induced malignant transformation of human cells.
While individual roles of elevated PKC isozymes in TCDD-induced transformation
still remain to be elucidated, it is of a particular interest to speculate that, since NIH3T3
cells overexpressing PKC-ε showed characteristics of malignant transformation similar
to those of TCDD-transformed cells [9], this particular isozyme could be one of
isoforms implicated with TCDD-induced carcinogenesis of human cells. In addition,
PKC is known to regulate calcium entry into the cell by phosphorylating ion flux
networks [28]. Overexpression of certain PKC isozymes, therefore, may be associated
with a lowered function of calcium mobilization from the extracellular space observed
Fig. 3. Agonist-stimulated cAMP production in parental cells (open bar) and TCDD-transformed cells (solid bar). *, p < 0.05 as compared to parental cells.
Fig. 4. Expression of PKC isozymes in parental cells (lane 1) and TCDD-transformed cells (lane 2). The lysate of mouse brain (lane 3) was used as a positive control. Conventional PKC isozymes, cPKC; novel PKC isozymes, nPKC; and atypical PKC isozymes, aPKC.
in the TCDD-transformed cells. Further studies on the spatial distribution of individual PKC isozymes will be required to clarify their roles in the carcinogenesis process.

Taken together, our findings suggest that alterations of PLC-coupled signals, adenyly cyclase-mediated responses and PKC isozyme expressions represent some of alterations in signaling pathways associated with the neoplastic transformation of this cellular system.

While several studies demonstrated mechanisms of TCDD-induced toxic responses including cancer (Yang and Rhim, 1995), no attempt has been made to study TCDD-induced carcinogenesis in human cells with respect to signal transduction pathways. Thus, the current study may provide a basis to investigate signal transduction pathway as cellular events that may lead to the elucidation of a mechanism of TCDD-induced carcinogenesis in human cells.

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