Human Epithelial Cells with Long-term Exposure to TCDD and Successive Cell Divisions Exhibit Alteration of Second Messenger Molecules

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Abstract

In our previous study, human cells exposed to TCDD for 2 weeks and subsequent 6 subcultures showed a malignant transformation. Effects of second messenger molecules in the transformed cells and the parental cells were assessed with respects to $[Ca^{2+}]_i$, IP_3, and cAMP. The present study demonstrated that, while constitutive elevation of IP_3 level was observed in the TCDD-transformed cells, induction of IP_3 production and intracellular free calcium concentration by extracellular signals such as ATP or histamine were 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. The results suggest that alteration of second messengers in human cells observed after long-term exposure of TCDD and successive cellular expansion may play roles in underatanding not only TCDD-induced carcinogenesis but fundamental basis of neurochemical regulation.

Introduction

TCDD is a widespread environmental contaminant, which is known to affect neurobehavioral and neurochemical effects[1]. While a variety of TCDD-related neurochemical effects are reported, information on the effects of second messengers and signal transduction with exposure to TCDD is limited. Changes of inositol phosphates or cAMP levels were reported in rodent models after acute exposure[2]. TCDD is known to alter calcium channel activity in primary rat astroglia cells and increase intracellular calcium levels after neuronal injury[3]. However, most of effects on second messengers ever reported are the consequence of short-term exposure. No one has ever reported possible changes of second messengers after the long-term exposure of TCDD and successive cellular division, which is more relevant to the real

environmental situation. Thus, the present study has attempted to analyze second messenger molecules in the TCDD-transformed human cells in an effort to understand effects of signal transduction pathway after long-term exposure of TCDD.

Experimental 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[4]. 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[5]. Growth and maintenance medium for these cell lines consisted of Dulbeco'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).

Chemical treatment: The parental cells or the transformed cells were treated with 100 μM ATP or 300 μM histamine in presence or absence of extracellular calcium (2.2 mM). To measure cAMP accumulation, [^{3}H]adenine-loaded parental cells or transformed cells were preincubated with IBMX (1mM) for 15 min and then stimulated with forskolin (10μM), PGE_2 (10μM) or isoproterenol (1 μM) for 20 min.

Measurement of Intracellular Free Calcium Level: Intracellular free Ca^{2+} concentration was determined using the fluorescent Ca^{2+} indicator fura-2 as previously reported[6]. Calibration of the fluorescence signal in terms of intracellular free calcium concentration was performed according to Grynkiewicz *et al*[7]. 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[5]. When cells were grown in six well culture plates up to 90% of the confluency, 100 μM ATP or 300 μM histamine were treated for 20 sec. IP_3 concentration in the sample was determined based on a standard curve and expressed as picomoles per microgram of protein. IP_3 binding protein was prepared from bovine adrenal cortex according to the method of Challiss *et al.*[8].

Measurement of [^{3}H]cAMP Formation: Intracellular cAMP was determined by measuring the formation of [^{3}H]cAMP from [^{3}H]adenine nucleotide pools as described previously by Salomon with some modifications[9]. The cells were grown in six-well plates to confluency and loaded with [^{3}H]adenine (2 μCi/ml) (NEN, Boston, MA) in complete medium for 24 h.r. Increases of intracellular cAMP concentration were calculated as [^{3}H]cAMP/([^{3}H]ATP + [^{3}H]cAMP) x 10^3.

Statistical analysis: Student t-test was used to compare individual means. The level of significance was $p < 0.05$.

Results

Treatment of 100 μM extracellular ATP induced higher stimulation of $[Ca^{2+}]_i$ in the parental cells than in the transformed cells. Increases of $[Ca^{2+}]_i$ by extracellular calcium was 2.4 fold higher in the parental cells than in the transformed cells.

Treatment of 300 μM histamine also showed higher stimulation of $[Ca^{2+}]_i$ in the parental cells. Increases of $[Ca^{2+}]_i$ by extracellular calcium was 1.9 times higher in the parental cells. (Fig. 1.) While steady state of IP_3 was higher in the transformed cells, stimulation of IP_3 formation by ATP or histamine was significantly lower in the transformed cells than in the parental cells. (Fig. 2.) Steady-state levels of cAMP accumulation 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 induced a lower level of cAMP accumulation in TCDD-transformed cells than in parental cells. Treatment of PGE_2 also showed a lower induction of cAMP accumulation in the transformed cells. However, isoproterenol treatment did not show a significant difference of cAMP accumulation between the two cell lines (Fig. 3.).

Discussion

When parental cells and TCDD-transformed cells were treated with ATP or histamine, 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 the mechanism of malignancy or neurochemical regulation after long-term exposure of TCDD.
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. While isopreterenol 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.

Taken together, our findings suggest that alterations of second messenger molecules may represent some of altered signaling pathways responsible for the neoplastic transformation of human cells and , possibly, regulation of neurochemical effects after long-term exposure of TCDD. While many studies demonstrated neurobehavioral and neurochemical effects of TCDD[1)], no attempt has been made to elaborate effects of second messengers and signal transduction pathways in human cell system following long-term exposure of TCDD and successive cell division.. Thus, the current study represents a first evidence of the alterations of a signal transduction pathway after a long-term exposure of TCDD and provides a basis to investigate signal transduction pathway as cellular events that may lead to elucidating the mechanism of TCDD-mediated neurochemical regulation in human cells.

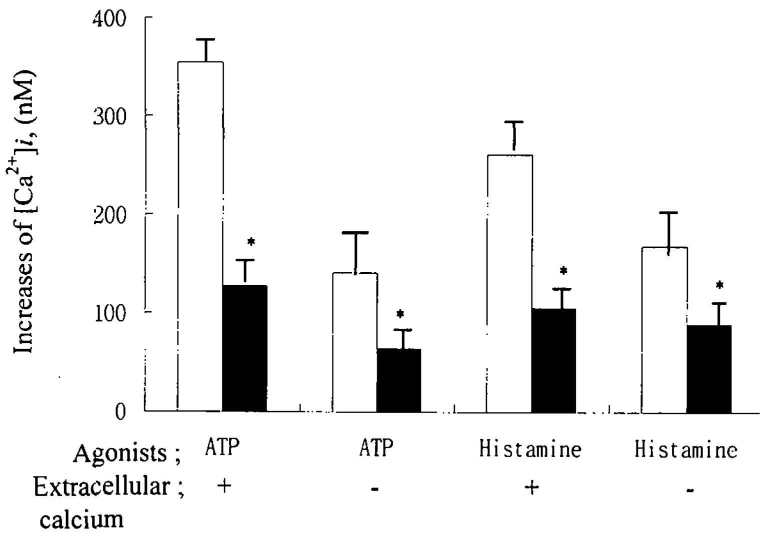

Fig. 1. Effects of ATP or histamine on increases of $[Ca^{2+}]i$ in the parental cells(□) and TCDD-transformed cells(■) * ; $P < 0.05$

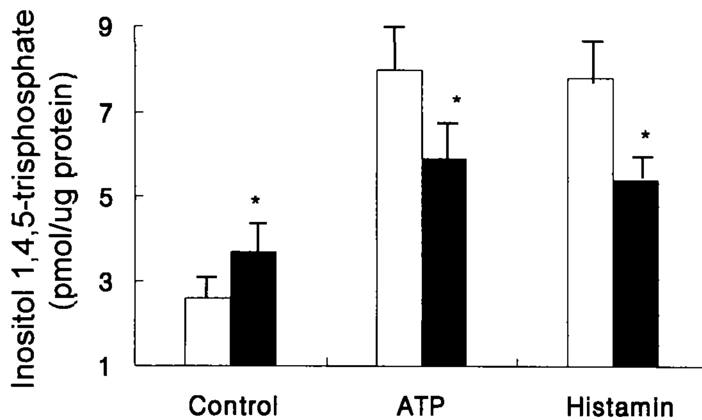

Fig. 2. Effects of ATP or histamine on IP_3 generation in parental cells(□) and TCDD-transformed cells(■) * ; $P < 0.05$

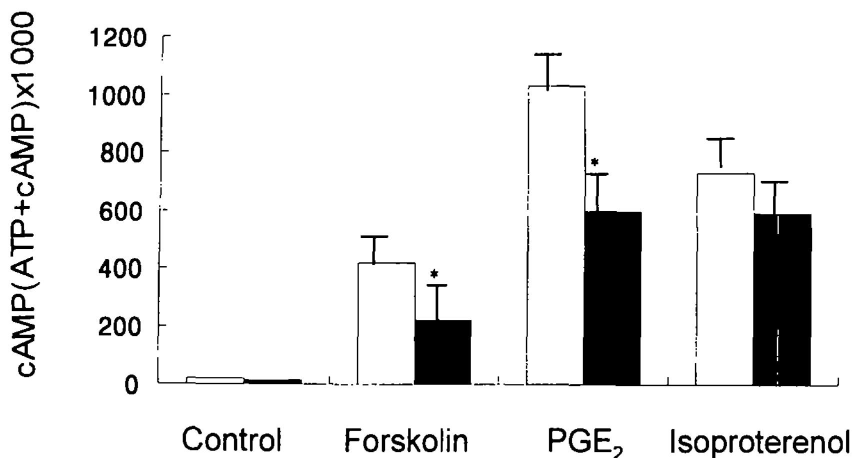

Fig. 3. Agonist-stimulated cAMP production in parental cells (□) and TCDD-transformed cells (■). * ; $P < 0.05$

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