MECHANISM OF ANTAGONISTIC ACTION OF TCDD ON INSULIN-INDUCED CELL PROLIFERATION SIGNALING IN MCF10A HUMAN BREAST EPITHELIAL CELLS

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Introduction

We have been interested in the effect of TCDD on the insulin signaling for sometime[1,2]. In brief, it appears that TCDD causes down-regulation of insulin receptor on the hepatic plasma membrane *in vivo*. To study the possible mechanism of TCDD's action on insulin's action *in vitro*, we must first establish a reliable *in vitro* model where the signaling of insulin is clearly blocked by TCDD.

After a search, we selected MCF10A immortalized human breast epithelial cells as the best study material. This cell line is well known for its response to insulin to proliferate even in the absence of serum so long as there is a trace amount of EGF[3]. The underlying mechanism of insulin's action through both insulin receptor and IGF receptors are well known. Furthermore, for our purpose MCF10A cells have been already studied by Scott Burchiel's group[4] to be highly responsive to TCDD with respect to increasing protein tyrosine kinase activities as well as mimicking insulin's action through the IGF – I receptor.

Materials and Methods

The MCF10A cells were obtained from the American Type Cell Culture Collection and were grown in complete growth medium (1:1 mixture of Ham's F12 Medium and Dulbecco's Modified Eagle's Medium with 2mM L-glutamine supplemented with 20 ng/ml epidermal growth factor (EGF), 100 ng/ml cholera toxin (CTX), 5 µg/ml insulin (I), 500 ng/ml hydrocortisone (H) and 5% calf serum bovine). The medium was renewed every 2-3 days. When the cells reached 70% confluency the medium was changed to MEBM (Mammary Epithelial Basal Medium, Serum-free) from Clonetics Cat no. CC3151, supplemented with 10 ng/ml epidermal growth factor (EGF), 1 µg/ml hydrocortisone (H) in a presence or absence of 100 ng/ml cholera toxin (CTX). After 24 h the same medium was replaced and the appropriate hormone or chemical (IGF-I, 2,3,7,8-TCDD, insulin or their combinations) were added.

For cell proliferation assay, MCF10A cells were placed in 6-well plates (Corning Glass) at $3.5x10^4$ cells/well in complete medium with a final volume 3 ml. The cells were allowed to stabilize and attach for 24 h at which time the medium was changed to serum-free (MEBM) supplemented with 10 ng/ml epidermal growth factor (EGF), 1 µg/ml hydrocortisone (H) plus or minus 100 ng/ml cholera toxin (CTX). The appropriate hormone (insulin or IGF-I), IGF-IRµ (1H7) antibody, 2,3,7,8-TCDD or their combinations were added. Cells were grown under treatment for 6 days. The medium was changed every other day and appropriate hormones, antibody or chemicals were added simultaneously.

For biochemical studies, whole cell extract was prepared as follows. The treated cells were rinsed twice with phosphate-buffered saline (PBS) and then lysed on ice with 200 µl per 100 mm dish cold RIPA buffer consisting of 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM Na_3VO_4 1% NP-40, 0.1% SDS, 0.5% Sodium deoxycholate and protease inhibitor cocktail (Sigma, 1:100 dilution) for 30 min. The resulting extract was clarified by centrifugation at 16,000 x g in a microfuge for 20 min at 4°C and was frozen at -80°C. Protein was determined using the Bio-Rad D_c protein assay.

Whole cell extract (30 µg/lane) proteins were separated by 10% SDS-PAGE at 100 V (constant), and Western blotting was carried out with primary antibody:antiphosphotyrosine, clone 4G10 (Upstate Biotechnology) at 1:10,000 dilution in blocking solution overnight at 4°C. After incubation with horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature, blots were developed using SuperSignal West Pico detection kit (Pierce, Illinois).

Results and Discussion

We first studied the effects of TCDD on cellular proliferation induced by insulin. Under the standard culturing condition (see "Methods"), addition of insulin always caused marked increase in proliferation of MCF10A cells, and in the co-presence of TCDD the extent of insulin-induced proliferation was significantly less (Fig. 1A). Such an antagonistic effect of TCDD against insulin could not be reversed by a specific antibody (Ab) against IGF-receptor. However, when cholera toxin (CTX) was deleted from the standard medium (Fig. 1B), the antagonizing action of TCDD disappeared, indicating that some cultural conditions may be very important in expressing such a property of TCDD. Since insulin is known to act as the ligand for both insulin receptor and IGF (insulin-like growth factor) receptor, we replaced insulin with IGF-I, which acts specifically on the latter receptor. The results showed that TCDD did not antagonize the proliferative action of IGF-I, (the result not shown). This set of data supports the hypothesis that the insulin antagonizing action of TCDD is likely mediated mainly through insulin receptor and not through IGF-receptor. In the next experiment we addressed the question on the mechanism of action of cholera toxin in affecting the action of TCDD (Fig. 2). MCF10A cells were grown on the standard medium with or without CTX. Cells were harvested, homogenized and proteins extracted as described in "Methods" and were analyzed for phosphotyrosine through Western Blotting with anti-phosphotyrosine antibody. The results showed that CTX has a potent suppressive action on overall protein tyrosine phosphorylation activities (data not shown). Therefore, it is possible that in the absence of CTX MCF10A cells show such high levels of tyrosine kinase activities that effects of TCDD to induce additional tyrosine phosphorylation are masked. Next, we studied the combined action of TCDD and insulin (Fig. 2 left) or TCDD and IGF-I (Fig. 2 right) using the same criterion. The result of the former experiment confirmed that TCDD rapidly stimulates tyrosine phosphorylation (see 15 min.). The action of insulin was also rapid but the difference from TCDD was that by 15 min. the intensity of major bands already decreased significantly. The combined action of TCDD and insulin, on the other hand, showed a significant increase, indicating that the above down-regulation phase of the action of insulin was clearly antagonized by TCDD. The repeat of the same experiment with IGF-I, in place of insulin, showed that down-regulating IGF-I is not as powerful as insulin in tyrosine phosphorylation at 15 min. Futhermore, the antagonizing action of TCDD on this process at 15 min. was less pronounced than the case of insulin, though somewhat similar tendencies were observed.

As for the likely mechanism by which TCDD could cause interference with insulin signaling, we view the action of TCDD on the early event of insulin-induced tyrosine phosphorylation and (5 min in our experiment) immediate dephosphorylation thereafter (measured

at 15 min.) to be the critical factor, since it has already been shown that the initial increase in tyrosine phosphorylation (5 min) and timely dephosphorylation is the essential requirement of successful signaling of insulin through insulin receptor[5].

With regard to the identity of the protein tyrosine kinase activated by TCDD at this early stage of its action, both C-SRC and the insulin receptor kinase must be considered as the primary candidates. The former has already been shown to be rapidly activated in several types of cells[8-10] and the latter must be involved, in this case at least, when both insulin and TCDD were added together (see Fig. 2 left). While the involvement of other growth factor receptor associated kinases cannot be ruled out, the possibility of their significant roles are less likely, since in this system, only insulin has been designed to play the stimulatory role on cell proliferation. Currently, experiments are being conducted to shed some light on this intriguing question.

As for the phosphatases involved, dephosphorylation of tyrosine phosphorylated proteins is known to be carried out by a few phosphatases. For example, both SHP1 and 2 are recruited to the vicinity of insulin receptor at the plasma membrane site upon insulin-induced activation of tyrosine kinases. Therefore these two are the most frequently cited phosphatases[6, 7], but the involvement of other phosphatases is also likely[5]. However, for the purpose of this work, the important point is that this timely dephosphorylation process is clearly hindered in cells treated with TCDD (Fig. 2 left).

Supported by grants ES05233, ES07284, and ES0570 from the National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina.

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