Significance of Ah-receptor Mediated Activation of Protein Kinase under Cell Free Conditions

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To understand the meaning of TCDD-induced increases in protein phosphorylation activities, we have made an attempt to search for the existence of a protein kinase mediated pathway for TCDD using *cell free* preparations from adipose tissue of male guinea pigs. The reasons for selection of this biological material are (a) adult male guinea pigs are the most susceptible animals to the lethal action of TCDD (LD50 = 0.6 μg/kg), (b) the reduction of its adipose tissue (i.e., "wasting syndrome") is one of its major toxic endpoints, and (c) TCDD at very low doses is known to cause rapid changes in protein phosphorylation (1) as well as glucose transporting activities in this tissue [2,3]. Unexpectedly, we have found that TCDD is capable of activating some protein kinases under strictly *cell free* conditions even without the nucleus. Such an effect of TCDD takes place very rapidly within 1 to 10 min, and is dependent on the Ah-receptor. Based on these observations we now propose an second major pathway, in addition to the known "XRE" e.g. pathway [4]. for the toxic action mechanism for this class of chemicals.

MATERIALS AND METHODS

Preparation of Cell Fractions — Epididymal and abdominal adipose tissues of untreated guinea pigs were homogenized in 3 volumes (w/v) of buffer A (10 mM 0.05 HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl and 0.05 mMDTT). The homogenates were centrifuged at 700 x g for 10 min at 4°C in a Sorvall SS34 rotor to pellet nuclei. The low speed (7000 x g) supernatant from this step was designated as the extranuclear fraction which was carefully decanted and either used in the assay as total extranuclear fraction or centrifuged for 60 min at 100,000 x g (Beckman Type 50 rotor) to obtain cytosol. The pellet (crude nuclei) was resuspended in 500 μl of buffer B (20 mM HEPES pH 7.9, 25% (v/v) glycerol., 042 M $NaCl_2$,1.5 mM $MgCl_2$, 0.2 mM EDTA, 05. MM PMSF, 0.5 mM DTT plus 10 μg/ml of the other protease inhibitors, leupeptin, aprotinin and chymostatin, all of which were added to the buffer just before the extraction) with a glass/glass homogenizer. The suspension was stirred gently with a magnetic stirring bar at 4°C for 30 min. and then centrifuged for 30 min. at 25,000 x g. The resulting clear supernatant was used as a nuclear extract.

Cell Free Phosphorylation Studies — For SDS-polyacrylamide gel electrophoresis (SDS-PAGE) studies, an aliquot containing 100 μg protein from each fraction (cytosol, micromosomal and nuclear fractions) was incubated with 10 nM TCDD *in vitro* at 30°C in 80 μl of 50 mM HEPES, pH 7.9, 10 mM $MnCl_2$, and 10 μM Na_3VO_4. After 10 min., 1 μCi (3 μM final) of γ-^{32}P-ATP was added, and the reaction was terminated after 60 sec. by addition of 40 μl 4X SDS-treatment buffer [3] and analyzed on 10% SDS-PAGE.

Protein Phosphorylation Assay Using Phosphocellulose Paper Method —A portion (20 μg protein) of 7000 xg supernatant was incubated in 80 μl of 50 mM HEPES, pH 7.9, 10 mM $MnCl_2$ and 40 μg histone with and without various test agents shown in Table 1, Experiment I. The reaction was performed in the presence and absence of 10nM of TCDD as before [3]. For protein tyrosine kinase assay a specific substrate peptide (RR-SRC, catalog number 3124A, from Gibco BRL) was used at 50 μM final concentration with 20 μg protein of 7000 x g supernatant. The phosphorylation with 0.5 μCi γ-^{32}P-ATP (3 μM ATP) was run for 60 sec.

Gel Mobility Shift Assay and Immunoprecipitation Study — was carried out by the method of Enan and Matsumura [3,5].

RESULTS AND DISCUSSION

In the first series of experiments we tested the effect of a direct addition of TCDD to isolated nuclei, cytosol and postnuclear membrane fractions (i.e., microsomal fraction) prepared from the homogenate of adipose tissue, all under *cell free* conditions. After incubation with 10 nM TCDD, the changes in protein phosphorylation activities were assessed[5] by reacting the incubated mixtures with γ-^{32}P-ATP, terminating the reaction with sodium dodecyl sulfate (SDS) and analyzing ^{32}P-phosphorylated proteins using SDS-polyacrylamide gel-electrophoresis, and quantitative radioscanning (data not shown). It was immediately noted that TCDD could stimulate protein phosphorylation activity *in vitro* under *cell free* conditions even in preparations lacking nuclei. Of 3 fractions tested separately or in combination, the TCDD-caused increase in phosphorylation was most prominent in cytosol, followed by a small increase in postnuclear membrane fraction. The overall protein phosphorylation activity in the nucleus was low under this experimental condition (every fraction tested at 100 μg protein per test). The dose-response curve of TCDD action on protein tyrosine kinase was studied next using 7000 xg preparation. The results showed that the stimulatory effect of TCDD is noticeable even at 1nM and becomes significant ($p \leq 0.01$) at the 10 nM range (data not shown).

To gain an insight into the basic mechanism of TCDD-induced activation of protein phosphorylation, the effect of two each of protein synthesis inhibitors and Ah-receptor blockers were tested using the same cell and nucleus free preparations with histone as an artificial substrate (Table, 1, Exp I). The results clearly showed that TCDD's stimulatory effect could be abolished by these Ah-receptor blockers at low enough concentrations, but not by actinomycin D or cycloheximide. These data are consistent with he idea that this aspect of TCDD's action is not mediated through *de novo* protein synthesis. To study the action of TCDD on a protein phosphorylation-dephosphorylation system under nucleus free conditions, we have tested the effect of a phosphatase inhibitor, okadaic acid, and two protein tyrosine kinase inhibitors on TCDD-induced changes in cytosolic phosphorylation activities on histone (Table 1, Exp II). Under these incubation conditions favoring protein tyrosine kinases (i.e., $MnCl_2$ 10mM, heparin 100 nM, EGTA 1 mM), the stimulatory action of TCDD was apparent by 10 min. As expected, okadaic acid added simultaneously with TCDD increased the level of phosphorylation on histone as compared to the control, but it did not eliminate TCDD's action. Genistein, an overall protein tyrosine kinase inhibitor inhibited the stimulatory action of TCDD, while bombesin, a known stimulator of protein tyrosine kinase slightly synergized. Heparin at this concentration is known to inhibit casein kinases [6]. It decreased the overall phosphorylation activity, but in the co-presence of either bombesin or okadaic acid, heparin did not antagonize the stimulatory effect of TCDD. These observations indicate the possible involvement of some protein tyrosin kinases elevating the protein phosphorylation activities in TCDD treated preparations.

To investigate whether such an action of TCDD to stimulate protein phosphorylation occurs at the cytosolic form of the Ah-receptor complex, the latter was isolated from 100,000 x g supernatant using the anti-Ah-receptor antibody and protein A/G sepharose, washed extensively and treated with TCDD (Note that in this experiment all agents including histone γ-^{32}P-ATP and other chemicals were added at the same time as TCDD). The results were essentially the same as those of Table 1, indicating that cell-free activation of protein kinases occur even in isolated Ah-receptor fraction (data not shown).

In the experiments summarized in Figure 1 the time course of TCDD response of four different types of protein kinase was examined. It is interesting to note that while the overall extent of stimulation by TCDD was highest in the case of protein tyrosine kinase(s) (i.e., approximately to 300% in 20 to 30 min.), the quickest responding one was MAP-2 kinase (20 to 25% increase in 1 to 2 min.). As for protein kinase C (PKC), there was an initial decline in the activity immediately after the addition of TCDD, but it was followed by a stimulatory phase which started at 20 to 30 min. cAMP dependent protein kinase responded to TCDD in a similar fashion as PKC.

The fact that MAP-2 kinase is elevated by TCDD's action raised the possibility that the signal

triggered by TCDD also activates the growth factor signal transduction pathway (7). This was tested by first reacting TCDD with isolated cytosol as before, combined with isolated nuclear proteins and running a gel-retardation assay [8] using a ^{32}P-labeled AP-1 binding DNA probe. AP-1 proteins are known to increase their overall DNA binding ability by increased phosphorylation through several protein kinases that are indispensable components of the growth factor signal transduction pathway [6]. Under this condition TCDD clearly stimulated AP-1 binding, with TCDD's stimulatory action most pronounced at the 5 min. incubation time point (Fig.2A). This stimulatory action of TCDD could be eliminated by simultaneous addition of genistein (data not shown). To confirm this observation on intact adipocytes, isolated adipose tissue slices were treated with TCDD in a tissue culture medium (2) and the same gel retardation assay was conducted yielding very similar results (Fig.2B).

In summary, we could clearly show that the initiation of some part of TCDD's action pathway does not require the presence of the nucleus, though as far as the activation of protein kinase activities is concerned, such a nuclear independent action pathway still requires the participation of the Ah-receptor as judged by the action of Ah-R blockers. Such a protein phosphorylation mediated action pathway could play a significant role in at least some of the toxic actions of dioxin-type chemicals [8]. In support of this hypothesis, we have previously shown that the toxic effect of TCDD *in vivo* to induce thymic involution in C57 mouse strain could be antagonized by quercetin, a known inhibitor of protein kinases [9]. Thus, this TCDD-induced cytosolic "protein phosphorylation pathway" must be considered as a separate route of action for dioxin-type chemicals from the well established nucleus translocation dependent pathway which activates various genes via "XRE" [4].

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Table 1. Effect of TCDD added under cell free conditions on protein phosphorylation in postnuclear fraction (i.e., 700 xg supernatant) from the homogenate of guinea pig adipose tissue. Note that histone was used as a general substrate protein in all tests.

Treatments	pmoles 23P/10μg histone/5 μg protein/60 sec ($\bar{x}$ ±SD)	
	Control	TCDD (10 nM)
Experiment I		
no addition	10.3 ±0.1	11.7 ±0.2**
actinomycin D (2 μg/ml)	10.3 ±0.3	11.7 ±0.4**
cycloheximide (0.4 μM)	10.4 ±0.3	11.7 ±1.0**
4, 7-phenanthroline (10 μM)	10.3 ±0.5	10.3 ±0.7
∝-naphthoflavone (1 μM)	10.1 ±0.7	10.1 ±0.6
Experiment II		
no addition	13.4 ±0.6	19.8 ±1.3**
heparin (100 nM)	14.5 ±0.7[a]	15.0 ±0.9
genistein (15 μM) + heparin	11.7 ±0.8[a]	15.2 ±0.8**
bombesin (10 nM) + heparin	15.5 ±1.0[b]	20.0 ±1.9**
okadaic acid (1 μM) + heparin	15.2 ±0.9[b]	18.3 ±2.0**

The data are the mean of 9 assays ±SD.** Significantly different from the corresponding control value ($P \leq 0.01$).[ab]Significantly different from no addition value (13.4 ±0.6) $P \leq 0.05$ or $P \leq 0.01$ respectively. (Cochran t-test was used in all statistical analysis)

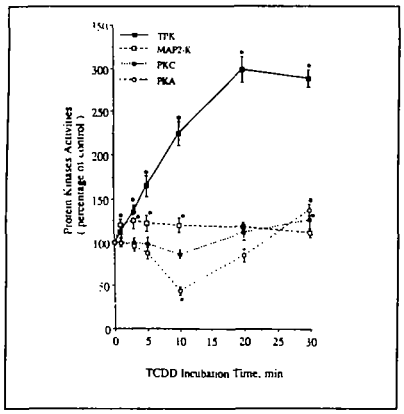

Fig. 1.
The time course of TCDD induced activation of individual protein kinases in the 7000 xg supernatant under cell free conditions. Phosphocellulose paper method was used to assay the kinase activity in all cases. The data are the mean of 9 independent assays ± SD. *Significantly different from control at P≤0.01 (Cochran t-test). The substrate peptides used are: RR-SRC (RRLIEDAEYAARG) for protein tyrosine kinase; AC-MBP (ACOKRPSQRSKTL) for protein kinase C; kemptide (LRRASLG) for protein kinase A; and APRTPGGRR for MAP kinase.

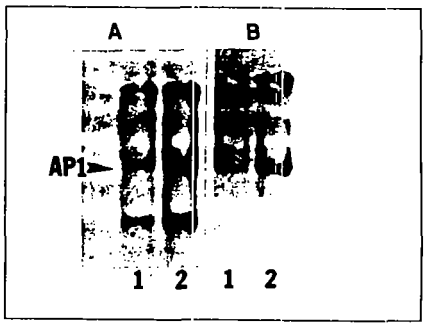

Fig.2 Gel-retardation assay on the effect of TCDD under cell and nucleus free condition on the abilities of isolated nuclear proteins to bind to a specific ^{32}P-labeled, AP-responsive element DNA

Panel A: The cytosol incubated with TCDD (10 nM) in *cell free* condition for 5 min (Lane 2), and the control sample (Lane 1) which received the same volume of the solvent (acetone) only. *Panel B*: Gel-retardation assay on the effect of TCDD on binding properties AP-1 proteins to AP-1 responsive element in intact adipocytes. For this test 1 g pieces of intact adipose tissues were isolated and maintained in DMEM medium gassed thoroughly with 95% O_2 and 5% CO_2 with 13.3 mM glucose (16,17). Each piece in 10 ml of medium was treated either with TCDD (to make 10 nM) or acetone (for control), and incubated for 1 h. DNA probe binding reaciton was run in 20 μl total volume with 5 μg nuclear protein form control (Lane 1) or TCDD-treated sample (Lane 2) under the 10 mM $MnCl_2$ condition.

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