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REGULATION BY 2,3,7,8-TETRACHLORODIBENZO-*p*-DIOXIN (TCDD) OF THE DNA BINDING ACTIVITY OF TRANSCRIPTIONAL FACTORS VIA NUCLEAR PROTEIN PHOSPHORYLATION IN GUINEA PIG ADIPOSE TISSUE

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Abstract—2,3,7,8-Tetrachloro-*p*-dioxin (TCDD) induced a modest stimulation of nuclear protein phosphorylation in explant tissue cultures in 10 min, followed by a substantial decrease in the level of total protein phosphorylation activity in the nucleus. Curiously, this TCDD-induced decline in nuclear protein phosphorylation was accompanied by an increase in cytosolic and extranuclear protein phosphorylation activity. One of the main causes for such a decrease in the protein phosphorylation activity in the nucleus appears to be related to some increase in protein phosphatase activities as judged by the counteractions of okadaic acid and Na_3VO_4 to the above effect. In addition, TCDD induced changes in nuclear protein kinase activities as well. Manganese-stimulated protein kinase was found to be the predominant type of nuclear protein phosphorylating activity affected by TCDD, with 60% of the total activity due to heparin-sensitive casein kinase II (CK II), a major nuclear protein kinase. The level of CK II activity in the nuclear protein preparation from adipose tissue of TCDD-treated guinea pigs (1 μg/kg) in the presence of 100 nM heparin was only 35% of the control value after 24 hr. In addition, TCDD was found to increase the protein kinase C and microtubule-associated protein 2 kinase activities as early as 15 min after treatment in isolated adipose tissues in culture. Under *in situ* incubation conditions with explant tissues in culture, TCDD rapidly enhanced the DNA binding activity of the transcriptional factor AP-1, whereas the same treatment reduced c-Myc DNA binding activity. Genistein, a specific protein tyrosine kinase inhibitor, abolished the stimulatory effect of TCDD on AP-1 binding activity, but not on DNA binding activity of c-Myc. Phorbol ester (TPA) increased the binding activity of AP-1 and c-Myc, as expected. However, TCDD in combination with TPA caused a slight reduction in binding activity of both transcriptional factors. On the other hand, in the presence of forskolin, the stimulatory effect of TCDD on AP-1 binding activity and the inhibitory effect on c-Myc were still apparent. Okadaic acid almost abolished the binding activity of c-Myc, whereas in combination with TCDD a stimulatory effect was found. These observations are consistent with the idea that TCDD regulates the DNA binding activity of AP-1 and c-Myc mainly through modulating their states of phosphorylation by altering protein kinase and phosphatase activities.

Key words: dioxin; AP-1; c-Myc; kinases, mobility shift; adipose tissue

TCDD† is known to be the most active congener of a large family of toxic chemicals, collectively known as dioxin-type chemicals or toxic halogenated aromatics [1]. These chemicals are known to cause qualitatively different symptoms in many species, and their toxicities vary vastly among species [2, 3]. Therefore, it has been very difficult to find a common biochemical mechanism by which they affect various biological systems.

In terms of lethality, the most sensitive species to the action of TCDD is generally acknowledged to be the male guinea pig, with the LD_{50} of a single oral treatment on the order of 0.6 μg/kg [4]. In this species, the most conspicuous toxic endpoint of TCDD poisoning is the "wasting syndrome," which is characterized by body weight loss, mostly involving the loss of adipose tissue, and by an increase in serum lipids [5, 6]. Curiously, TCDD-triggered induction of the liver microsome systems, including cytochrome P450s, which is so prominent in other species, is not apparent in guinea pigs [2]. In view of the extreme sensitivity to TCDD in terms of the loss of adipose tissue in this species, we have chosen this biological material for our studies on the mechanism of action of TCDD [7–9]. Our studies have been aided by the development of an *in situ* (explant tissue culture) incubation method, using pieces of isolated but intact adipose tissue immersed in tissue culture medium. Addition of TCDD to this *in situ* system clearly causes some of the same changes observed *in vivo* [10, 11], including a sharp rise in total protein phosphorylation activity in the cytosol and the combined microsomal-plasma membrane fraction [12]. It is emerging now that protein phosphorylation regulates a diverse range of cellular processes [13]. The regulation of cellular programs through protein phosphorylation of key nuclear proteins is a growing area of interest. Among these proteins are the transcriptional factors, such as CREB, Jun, Fos, NF_KB and Myc. Some of these proteins respond directly to external stimuli such as those induced by growth factors and hormones, whereas others are phosphorylated cyclically in response to the timing mechanism of the cell cycle [14–17]. In the current work, we have extended our studies of the effects of TCDD on protein phosphorylation to those occurring on nuclear transcrip-

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† Abbreviations: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; DMEM, Dulbecco's modified Eagle's medium; CK II, casein kinase II; EMSA, electrophoretic mobility shift assay; TPA, phorbol ester; MAP, microtubule-associated protein; cAMP, cyclic AMP; PKC, protein kinase C; DTT, dithiothreitol; and MOPS, 4-morpholinepropanesulfonic acid.

tional factors. We found that TCDD initially caused a modest stimulation, followed by a substantial decline in overall protein phosphorylation activity within the nucleus, and that this effect was associated with stimulation of the binding activity of the transcriptional factor AP-1.

MATERIALS AND METHODS

Chemicals

[γ-^{32}P]ATP (~3000 Ci/mmol) and [^{32}P]H_3PO_4 (200 mCi/mmol) were purchased from Amersham (Arlington Heights, IL). D-Glucose and all other biochemicals were purchased from the Sigma Chemical Co. (St. Louis, MO). DMEM powder (Cat. No. 430-3800 EB) was purchased from Gibco (Grand Island, NY). The anti-rat MAP kinase R2 (rabbit polyclonal IgG) was purchased from UBI (Lake Placid, NY).

Animals

Four- to six-week-old (200–225 g), male English short hair guinea pigs (*Cavia porcellus*) were used throughout the study. Animals were housed in suspended stainless steel cages and provided with food and water *ad lib.* All animals were maintained on a 12-hr light/12-hr dark cycle at constant temperature (72 ± 1°F) and humidity (70%). *In vivo* treatments were made by a single i.p. injection of TCDD (1 μg/kg, 24 hr) in a corn oil:acetone vehicle (9:1); control animals received an equal volume of the vehicle only. Conditions for *in situ* explant cultures of adipose tissue were exactly as described by Enan *et al.* [10–12]. Animals were killed between 8:00 and 9:00 a.m., and their adipose tissues were removed rapidly, rinsed in cold normal saline, and chilled on ice. Three animals were used for each experiment.

In vivo *studies on nuclear protein kinases*

Adipose tissue was removed from untreated and treated animals 24 hr after a single i.p. injection of 1 μg TCDD/kg. The nuclear fraction was prepared [18, 19] and sonicated for 3 min on ice. Kinase activities were studied under the optimal conditions for each type. Manganese-dependent, calcium-calmodulin-dependent, and cAMP-dependent protein kinases were assayed as described by Enan and Matsumura [12, 20] using polyacrylamide gel electrophoresis. PKC was assayed according to Akers *et al.* [21]. For CK II, activity was measured as described by Lin *et al.* [22]. Briefly, the nuclear proteins were extracted from nuclear fraction with 0.35 M NaCl and 1 mM EDTA [18, 19], and samples of nuclear protein extracts (20 μg protein) were incubated with different concentrations of heparin in 100 μl of the reaction buffer (50 mM HEPES, pH 7.6, 150 mM NaCl, 10 mM $MgCl_2$ and 1 mM DTT). After 10 min of incubation at 30°, the phosphorylation reaction was initiated by adding 50 μg α-casein as substrate and 0.5 μCi [γ-^{32}P]ATP (1 μM final concentration), and terminated after 60 sec by adding 1 mL of tricholoroacetic acid (TCA, 20%) and 100 μL BSA (1 mg/tube). The tubes were kept on ice for 5 min, followed by centrifugation at 3000 *g* at room temperature for 3 min. The precipitate was dissolved in 100 μL of 1 N NaOH and reprecipitated with TCA/BSA. This step was performed three more times [23, 24]. The washed precipitate was finally dissolved in 200 μL of liquid scintillation solution, and the entire volume was transferred into 4 mL of liquid scintillation solution (Aquafluor, E.I. DuPont–New England Nuclear), and counted. The results were summarized as the means (±SD) of nine assays, calculated as fmol phosphorylated/50 μg casein/60 sec.

In situ *phosphorylation*

A piece of epididymal and abdominal adipose tissue (1.0 g) from an anesthetized (diethylether) untreated guinea pig was incubated for 8 hr with 0.25 mCi [^{32}P]H_3PO_4 in 10 mL DMEM (gassed thoroughly with 95% O_2, 5% CO_2) containing 13.3 mM D-glucose. TCDD (10 nM) was added at different time points during the labeling period. Controls received the same volume of vehicle (acetone) alone. The methods of Cronrath *et al.* [18] and Dignam *et al.* [19] were adopted for isolation and extraction of nuclear protein, with minor modifications. Briefly, the tissue was homogenized with 3 vol. of STM buffer (0.25 M sucrose, 20 mM Tris, 1.1 mM $MgCl_2$, pH 7.8) in a teflon/glass homogenizer at 4°. The homogenate was centrifuged at 2000 *g* for 15 min, the fat layer was removed, the pellet and the infranatant fluid were mixed and then filtered through a layer of cheesecloth, and the filtrate was centrifuged again at 4000 *g* for 15 min. The pellet was washed twice with STMG buffer (STM buffer containing 8.5% glycerol and 0.5% Triton X-100) and washed twice with STMG buffer without Triton X-100. The nuclear protein was extracted with 0.35 M NaCl and 1 mM EDTA and used in our studies.

An 80-μL aliquot containing 100 μg protein from each treatment was mixed with 40 μL of 4× SDS-treatment buffer [12] and heated in a water bath at 90° for 3 min. The entire volume of each tube was transferred into a well of 7% SDS polyacrylamide gel, which was subjected to electrophoresis and developed at a constant current (0.05 A) for 4–5 hr. The gel was stained and then destained, and the dried gel was scanned using a computerized radioactivity scanner (Ambis).

In another set of experiments without [^{32}P]H_3PO_4 labeling, three kinase activities were measured in the nuclear extract using specific substrate peptides and the optimal conditions for each. Substrate peptides for tyrosine kinase (RR-SRC), casein kinase II (Casein-α), and PKC (ACQKRPSQRSKTL) were purchased from GIBCO BRL. In all cases the phosphorylation reactions were carried out in the presence and absence of the substrate peptide, and the difference was adopted as the level of the specific phosphorylation by the kinase intended for the study. The tyrosine kinase assay was run exactly as described by Enan and Matsumura [25].

MAP2 kinase immunoprecipitation assay

Since MAP kinase phosphorylates a range of endogenous intracellular substrates, including nuclear proteins such as Jun and Myc [13], we had to first isolate MAP2 kinase through immunoprecipitation from *in situ* treatment of explant adipose tissue of guinea pig with anti-rat MAP kinase R2 antibody (rabbit polyclonal IgG). The nuclear fraction was prepared from adipose tissue already treated for 15 and 60 min with 10 nM TCDD or the vehicle alone (5 μL/5 mL medium). A 50-μL aliquot containing 100 μg protein from each treatment was mixed with 5 μg protein equivalent of MAP kinase R2 antibody and 50 μL of RIPA buffer (50 mM Tris, 150 mM NaCl, 1% Nonident P-40, 0.25% sodium deoxychloate, 2 mM EGTA, 1 μM aprotinin, 1 μM leupeptin,

pH 7.4). After a 1-hr incubation at 2°, 15 μL of protein A/G plus agarose was added to each sample and 30 min later the precipitate complex was washed three times with 1 mL of 20 mM Tris, pH 7.4, 150 mM NaCl and resuspended in 100 μL of MAP kinase assay buffer [26] (12.5 mM MOPS, pH 7.2; 7.5 mM $MgCl_2$, 0.5 mM EGTA, 0.05 mM NaF, 2 mM DTT and 0.05 mM Na_3VO_4). A 20-μL aliquot from each sample was incubated with a 50 μM concentration of a MAP2 kinase peptide substrate (APRTPGGPR, purchased from UBI) in 40 μL final volume of the assay buffer. All preincubation treatments were carried out at 0°. The kinase reaction was initiated by the addition of [γ-^{32}P]ATP (0.5 μCi, 1 μM) at 30° and stopped after 60 sec by spotting each 20-μL aliquot onto a piece of phosphocellulose paper. It was washed three times and counted as described above.

EMSA

TCDD (10 nM) was added to 1 g of guinea pig adipose tissue (explant tissue culture) with 5 mL DMEM. The tissue was incubated in the presence and absence of cycloheximide (500 μM), genistein (15 μM), forskolin (100 μM), okadaic acid (1 μM) and TPA (1 μM). After 15 min, the nuclei were prepared and extracted as described above. Protein concentrations were measured using the Protein-Assay Kit from Bio-Rad, and extracts were adjusted to 1 μg/μL. Transcriptional factors (AP-1) and c-Myc binding to DNA were measured by EMSA as described by the suppliers of the oligonucleotides (Stratagene). In brief, protein/^{32}P-labeled DNA complexes were formed at 4° for 20 min in 20 μL of reaction buffer containing 50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 5 mM DTT, 5 mM EDTA, 20% glycerol, 0.4 mg/mL sonicated salmon sperm DNA, and 5 μg of nuclear protein extract. A ^{32}P-labeled olignucleotide probe (50,000 cpm) (double-stranded AP-1 response element DNA, CTAGTGATGAGTCAGCCGGATC or double-stranded c-Myc DNA, CAGAGCACGTGGTCATG) was added and incubated at room temperature (25° ± 2) for an additional 20 min. Two microliters of 0.1% bromophenol blue in 70% glycerol was added to each sample, and the complexes were analyzed on 6% polyacrylamide gels (29:1 acrylamide:bis) in 0.25× TBE (Tris/borate/EDTA buffer).

RESULTS

In vivo *studies*

The data in Fig. 1 demonstrate that the *in vivo* treatment of TCDD for 24 hr reduced the overall level of nuclear protein phosphorylation under conditions that reveal the activities of several types of protein kinases (e.g. calcium-calmodulin, cAMP, calcium/phospholipid, and manganese-stimulated). In this nuclear preparation, manganese-stimulated protein kinases were the most active type of enzymes (lane 9). As expected, when Na_3VO_4 was omitted from manganese-stimulated kinase buffer, a further decrease in phosphorylation was observed (lanes 11 vs 9). This indicates that some active protein phosphatases are present in this fraction. Nevertheless, even without Na_3VO_4, TCDD (lane 12) still reduced the protein phosphorylation to a level markedly lower than that found in the corresponding control (lanes 12 vs 11). Cytosolic and microsomal fractions were prepared from the same *in vivo* treated and untreated tissues,

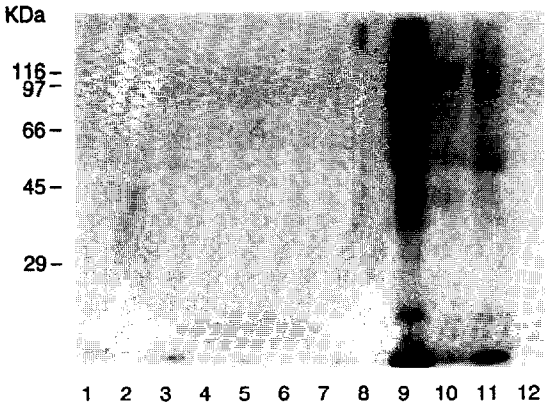

Fig. 1. Autoradiograph representing *in vivo* effects of TCDD on the activities of four nuclear protein kinases of guinea pig adipose tissue. TCDD (1 μg/kg) was administered as a single dose i.p., and animals were killed after 24 hr. The nuclear fraction was prepared and phosphorylated under different conditions with [γ-^{32}P]ATP, as described in Materials and Methods. Samples were phosphorylated under the following conditions: lanes 1 and 2 in buffer containing 10 mM $MgCl_2$; lanes 3 and 4 in the presence of 0.3 mM $CaCl_2$ and 25 nM calcium/calmodulin; lanes 5 and 6 in the presence of 10 mM $MgCl_2$ and cAMP (10^{-6} M); lanes 7 and 8 in the presence of phosphatidylserine, dioctanylglycerol and $CaCl_2$ (5 μg, 2 μg and 1.83 mM/tube respectively); lanes 9 and 10 in the presence of 10 mM $MnCl_2$ and 10 μM Na_3VO_4; lanes 11 and 12 same as 9 and 10 but without Na_3VO_4. Lanes 1, 3, 5, 7, 9 and 11 represent the control values. Lanes 2, 4, 6, 8, 10 and 12 represent the corresponding TCDD-treated ones.

and were analyzed simultaneously for their protein phosphorylation activities (Fig. 2). The results showed that TCDD caused an increase in extranuclear manganese-stimulated protein kinase activity in cytosol and microsomal fractions, in contrast to the reduction in the nuclear manganese-stimulated protein kinase activities.

One of the main questions raised by the above work was the identity of the major nuclear protein kinase(s) affected by TCDD. The results of the above experiments demonstrated that magnesium- and manganese-stimulated protein kinases are the predominant enzymes in the nuclei. When casein was used as a substrate under conditions favoring CK II, TCDD treatment was found to cause a significant reduction in the activity of this enzyme as compared with the control activity (Table 1). Heparin, a specific inhibitor of CK II at 100 nM, inhibited activity to 40% of control values of the manganese-stimulated nuclear protein kinase. At this dose of heparin, the value of TCDD-treated samples became almost the same level as that of control samples (Table 1), suggesting that CK II is one of the major kinases markedly suppressed by TCDD in the intact nucleus.

In situ *studies*

In view of the complex nature of the action of TCDD *in vivo*, explant cultures of guinea pig adipose tissue [12] were used throughout the subsequent investigation to study the direct effect on TCDD on phosphorylation of nuclear proteins without the influence of changing levels of hormones and growth factors in the blood. The results (Fig. 3) indicated that TCDD stimulated nuclear protein phosphorylation in 10 min followed by progressive suppression. The degree of suppression in the later phase

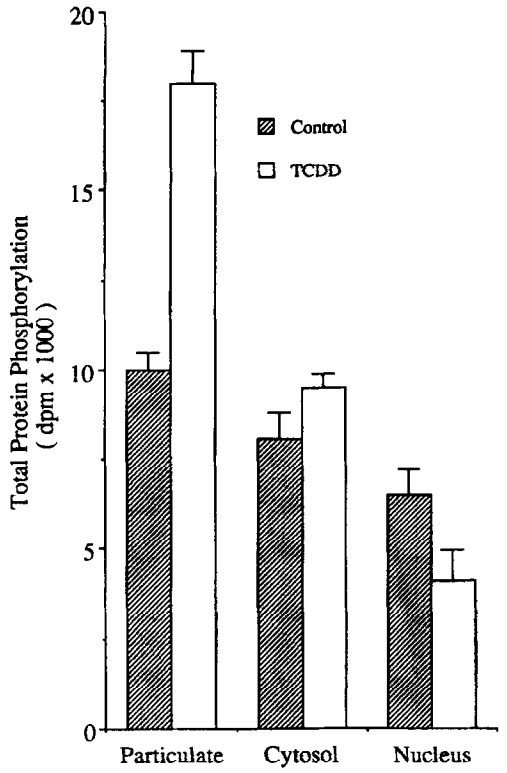

Fig. 2. Histograms representing *in vivo* action of TCDD on phosphorylation in nuclear, microsomal and cytosol fractions. For this study the animals were treated with 1 μg TCDD/kg for 24 hr (control animal received the same volume of the vehicle only) followed by collecting the adipose tissue. Particulate (microsomal), nucleus, and cytosol fractions were prepared by centrifugation and phosphorylated under manganese-dependent conditions using [γ-^{32}P]ATP as described in Materials and Methods. The ^{32}P-phosphorylated proteins (100 μg/lane) were electrophoresed (SDS–PAGE), and the gels were scanned using a computerized image scanner (Ambis). Data are the means ± SD of three different experiments, each run in triplicate.

Table 1. *In vivo* effect of TCDD on CK II in nucleus of guinea pig adipose tissue*

Heparin concentration (nM)	CK II activity (fmol ^{32}P/50 μg casein/60 sec)		
	Control	TCDD	TCDD-sensitive CK II† (%)
0	648 ± 75	350 ± 34‡	
50	427 ± 56	249 ± 20‡	45
100	263 ± 32	217 ± 7‡	35

* The treated animals were killed after 24 hr of i.p. treatment with 1 μg/kg TCDD. The control animals received the same volume of the vehicle only (corn oil:acetone, 9:1). Adipose tissue was removed, the nuclear proteins were extracted, and the assay was performed as described in Materials and Methods. Data are the means ± SD of three different experiments, each run in triplicate.

† TCDD-sensitive CK II activity was calculated as the difference between the values of the enzyme activity in TCDD-treated samples alone and TCDD-treated samples plus heparin. This difference was divided by the difference of the enzyme activity between control values and heparin alone, i.e. TCCD-sensitive CK II activity % = (350 − 217/648 − 263) × 100 = 35%.

‡ Significantly different from the control value ($P \leq 0.01$) (Cochran *t*-test).

increased with time, becoming considerable after 120 min of incubation with TCDD. These data are similar to an *in vivo* experiment that showed that administration of 1 μg/kg of TCDD caused a reduction in total nuclear phosphorylation levels (tested after 24 hr) in adipose tissue (Fig. 1).

In our preliminary study we found that, under *in situ* conditions, TCDD induced an increase in the phosphorylation of endogenous nuclear proteins under MAP2 kinase assay conditions as early as 15 min, even though no substrate peptide was added. The background values were 6.06 ± 0.08 and 7.40 ± 0.11 pmol/10 μg protein/60 sec for untreated and TCDD-treated explant culture of guinea pig adipose tissue, respectively. Because these conditions are also favorable to some other kinases, and because there are known endogenous protein substrates for MAP2 and CK II in the nucleus, we decided to first isolate MAP2 kinase using a specific antibody and assay its activity using a specific substrate peptide to directly study the effect of TCDD treatment on MAP2 kinase activity. Indeed, the data showed that in 15 min TCDD treatment (10 nM) significantly increased the MAP2 kinase activity as compared with control values (Table 2). However, such an increase appears to be transient, since by 60 min the level of MAP2 kinase activity in the treated tissues decreased to a level below the control.

Figure 4 shows the time–course of TCDD-induced changes in the activities of three nuclear kinases. TCDD (10 nM) induced the activity of PKC as early as 15 min, and this stimulatory effect was marked after 30 min of treatment. On the other hand, CK II activity was reduced after 15 min, and TK activities were lower after 30 min. Changes in CK II continued for the duration of the experiment (60 min), whereas the others were restored to their control levels after 60 min of treatment (Fig. 4).

With regard to the question of the relative importance of kinases versus phosphatases, the data obtained in Table 3 indicate that TCDD affects both protein kinases and phosphatases in nuclear protein extract. Both okadaic acid and vanadate were effective in raising the total level of protein phosphorylation, indicating the presence of active phosphatases. The fact that, in the presence of the tyrosine kinase-specific inhibitor, genistein, the effect of vanadate was minimal or absent supports the view that a significant part of TCDD-induced nuclear protein phosphatases are protein tyrosine phosphatases. It must be noted that the substrate peptide (RR-SRC) used here is known to favor Src-type protein tyrosine kinases. The sensitivity of the above enzyme assay system to this low dose of genistein attests to this interpretation. One possible candidate for protein tyrosine phosphatase is cdc 25, the major nuclear protein tyrosine phosphatase, which is known to act as a rate-limiting mitotic activator playing an antagonistic role to tyrosine phosphorylation of cdc 2, both of which are controlled by cyclin B [27]. However, more research would be needed to establish this point.

Finally, we asked the question whether TCDD could modulate the DNA binding activity of nuclear transcription factors AP-1 and Myc by changing their states of protein phosphorylation. We addressed this question using EMSA for AP-1 and c-Myc binding to AP-1 and

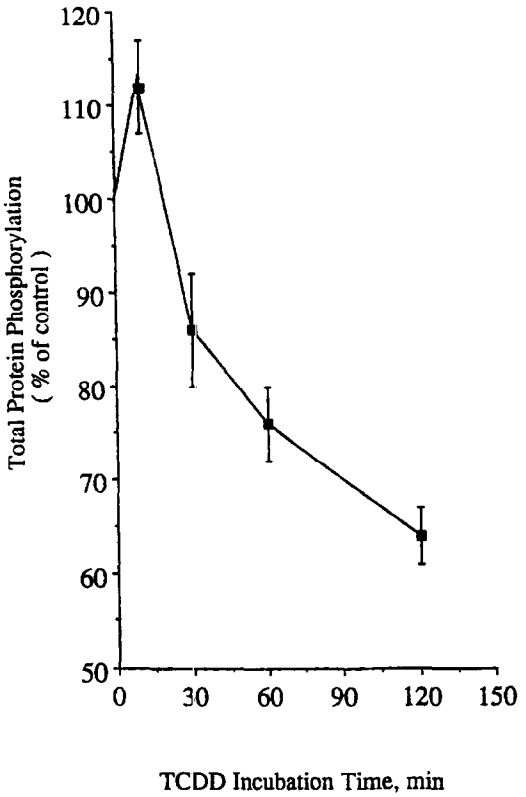

Fig. 3. Time–course of TCDD (10 nM) action on nuclear protein phosphorylation in isolated *in situ* guinea pig explant adipose tissue. The electrophoresed gel was dried and scanned using a computerized image scanner (Ambis). To obtain ^{32}P-phosphorylated samples, a piece of adipose tissue (1.0 g) from an untreated guinea pig was incubated for 8 hr with 0.25 mCi $[^{32}P]H_3PO_4$ in 5 mL DMEM (bubbled thoroughly with 95% O_2, 5% CO_2). TCDD was added at different time intervals during the labeling period. The adipose tissue samples were homogenized, and nuclei were collected by centrifugation and dissolved in an SDS buffer solution. Labeled nuclear proteins were analyzed by SDS–PAGE. Data are the means ± SD of three different experiments, each run in triplicates. The control value was 8500 ± 400 dpm.

c-Myc response element DNA. Explant cultures were treated with TCDD (10 nM) and/or other diagnostic chemicals for 15 min, and nuclear proteins were extracted and analyzed by EMSA. It was found that TCDD increased the binding activity of AP-1 (Fig. 5) and reduced DNA-binding activity of c-Myc (Fig. 6). Cycloheximide, a translation blocker, did not affect the stimulatory effect of TCDD on the binding activity of AP-1, whereas the effect of TCDD was abolished by treatment with genistein (Fig. 5). In the presence of forskolin and okadaic acid, the stimulatory action of TCDD on AP-1 binding activity still occurred. As expected, TPA alone increased the binding activity of AP-1, but in combination with TCDD a slight reduction of the binding was observed (Fig. 5). Genistein antagonized the inhibitory action of TCDD on c-Myc. Forskolin and TPA increased c-Myc binding, whereas okadaic acid reduced it. None of these chemicals was able to antagonize completely the inhibitory effect of TCDD on c-Myc binding to DNA (Fig. 6). Also, it was evident that the lower band in Fig. 5 is attributed to the AP-1 specific binding protein judging by a competition assay with excess AP-1 response element DNA. The upper band did not disappear in the presence of the same competitor; therefore, it appears to be due to a non-specific binding protein.

Table 2. *In situ* effect of TCDD on MAP2 kinase in nuclear fraction from explant culture of guinea pig adipose tissue*

Time of treatment	MAP2 kinase activity (pmol ^{32}P/1 nmol substrate/10 μg protein/60 sec)
15 min	
Control	1.081 ± 0.004
TCDD	1.350 ± 0.018†
60 min	
Control	1.163 ± 0.008
TCDD	1.035 ± 0.002

* MAP2 kinase of nuclear fraction was isolated using an anti-MAP kinase antibody as described in Material and Methods. The phosphorylation was performed as described by Clark-Lewis *et al.* [26] in the presence and absence of a 50 μM concentration of substrate peptide (APRTPGGPR). The values presented in this table are the differences of phosphorylation in the presence and absence of the substrate peptide. Data are the means ± SD of three different experiments, each run in triplicate.

† Significantly different from control value ($P \leq 0.01$) (Cochran *t*-test).

DISCUSSION

In the current study, we found that TCDD causes a transient stimulation of overall phosphorylation on nuclear proteins at an early stage, which is followed by a steady reduction both *in vivo* and *in situ*. The importance of such effects of TCDD on phosphorylation is apparent by the consideration of the central roles these protein kinases and phosphatases play in transmitting cellular signals, particularly growth factor signals, to nuclear transcription factors [28]. It has been generally acknowledged that the transmission of cellular regulatory signals, such as mitotic signals transduced from a growth factor, are processed first through the cytoplasmic signaling pathways in which protein kinases are important components [13, 28]. Further evidence suggests that, in the case of signals involving changes in gene expression, communication between the cytoplasm and nucleus relies on the changes in signal-dependent phosphorylation/dephosphorylation of nuclear transcription factors [13, 29, 30]. As far as it is known, upon TCDD binding to its cytosolic Ah-receptor, the receptor detaches itself from a complex with hsp 90, translocates to the nucleus, where it forms a dimer with another helix-loop-helix protein "arnt" (a nuclear transcriptional factor) and eventually interacts with the promoters of specific genes [31–33]. It has been demonstrated clearly, as the result of efforts of many scientists, that all of the active congeners belonging to the family of "dioxin-type" chemicals show significant affinities for the Ah-receptor, and that their interactions with this cytosolic receptor are the essential first step in the resulting toxic actions [2, 3].

One of the protein kinases decreased in the early stage of the action of TCDD is the nuclear CK II (Fig. 4 and Table 1), a widely distributed enzyme that phosphory-

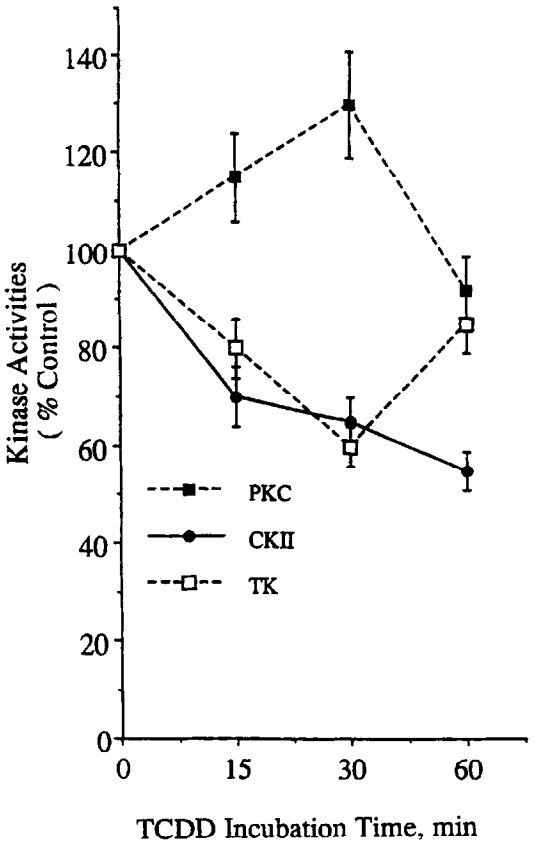

Fig. 4. Time–course of TCDD (10 nM) action on nuclear protein kinases. A piece of adipose tissue (2.0 g) from an untreated guinea pig was incubated with 10 nM TCDD in 5 mL DMEM for 15, 30 and 60 min. At each time point the tissue was removed, and the nuclear protein extract was prepared as described in Materials and Methods. Three nuclear protein kinase activities were assayed using specific substrate peptide for each one (see Materials and Methods). Abbreviations: PKC (protein kinase C), CK II (casein kinase II), and TK (protein tyrosine kinases). Data are the means ± SD of five assays. Control values were: CK II 595 ± 85 fmol ^{32}P/nmol substrate/5 μg protein/60 sec; PKC 3.5 ± 0.6 pmol ^{32}P/nmol substrate/5 μg protein/60 sec; TK 4.6 ± 0.5 pmol ^{32}P/nmol substrate/5 μg protein/60 sec.

Table 3. Effects of phosphatase and kinase inhibitors on nuclear protein phosphorylation

	Phosphorylation on RR-SRC peptide (pmol ^{32}P/1 nmol substrate/5 μg protein/60 sec)	
	Control	TCDD
No addition	14.3 ± 0.8	11.2 ± 0.9*
Okadaic acid (100 nM)	19.5 ± 1.0†	13.5 ± 1.1*‡
Na_3VO_4 (10 μM)	18.6 ± 1.1†	14.9 ± 0.8*‡
Genistein (5 M)	10.1 ± 0.6†	10.7 ± 0.6
Genistein + Na_3VO_4	10.4 ± 0.8†	11.0 ± 0.9

Nuclear protein was prepared from *in situ* TCDD-treated (i.e. in cultured medium with explant tissue) and untreated adipose tissue for 1 hr. Control samples received the same volume of the vehicle alone. The effects of different kinase and phosphatase inhibitors were tested in a reaction mixture described im Materials and Methods. Note that in this series of experiments no background values were subtracted from the above figures. The background value obtained in the absence of the synthetic substrate peptide, RR-SRC, was 10.1 ± 0.6 for both control and TCDD-treated samples. Data are the means ± SD of three different experiments, each run in triplicate.

* Significantly different from the corresponding control values, P ≤ 0.091 (Cochran *t*-test).

† Significantly different from no addition (control value 14.3 ± 0.8), P ≤ 0/01.

‡ Significantly different from no addition (TCDD alone value 11.2 ± 0.9), P ≤ 0.01.

lates a number of regulatory proteins in the cytoplasm and in the nucleus [34]. In the case of c-Jun, CK II is known to phosphorylate at its C-terminal site [20, 28, 34, 35]. This phosphorylation is regarded as a suppressive factor for its transcription activator activity. Thus, a decrease in CK II induced by TCDD could well cause functional activation, at least in theory. While we did not obtain the experimental evidence for the reduction of C-terminal phosphorylation, such an effect of TCDD on CK II at least provides an explanation for the observations in the current study. For example, Lin *et al.* [22] have reported that CK II is the major nuclear enzyme that phosphorylates Thr 231 and Ser 249 in the DNA-binding domain of c-Jun.

Since c-Jun is a required partner of dimerization with c-Fos and other AP-1 proteins, the increase in c-Jun activities is expected to elevate overall AP-1 binding activities [30]. Two potential complications to the above simplistic explanation may be the unknown nature of the protein phosphatase dephosphorylating c-Jun and the role of glycogen synthetase kinase 3 (GSK3) on c-Jun inactivation. Thus, caution must be exercised in interpreting the above data on decreased CK II only. On the other hand, the role of CK II on c-Myc functions appears to be relatively simple [13, 29, 30]; it multiplies, phosphorylates and activates all c-Myc proteins. Therefore, the TCDD-induced suppression on CK II is expected to cause a reduction in c-Myc binding to DNA, which is precisely the end result we obtained. However, we have not actually proven the sites or changes in the level of phosphorylation on any of these nuclear transcription factors. Furthermore, some of them are known to interact with other factors. Therefore, it appears prudent to be cautious in interpreting the above data.

Evidence suggesting that protein phosphorylation is involved in the regulation of AP-1 activity comes from the observation that the positive effect of AP-1 binding sites on transcription of linked genes could be increased by exposure of cells to TPA [36, 37]. In the current work, both TCDD and TPA induced the changes in binding activity of AP-1 (Fig. 5). TPA is a potent stimulator of PKC, a key mediator of intracellular signaling. TCDD also was found to stimulate the activity of PKC as early as 15 min after administration (Fig. 4). Furthermore, in the presence of TPA, TCDD did not cause additional stimulation on AP-1 binding, indicating that their action sites are the same or mutually interacting. In addition, in the current work, TCDD induced stimulation of AP-1 activity, and this effect was not antagonized by cycloheximide (Fig. 5), indicating that the early effect of TCDD does not require *de novo* protein synthesis. This agrees with what we have found previously [25, 38] regarding the effect of TCDD on protein phosphorylation under *cell-free* conditions. It is becoming very ap-

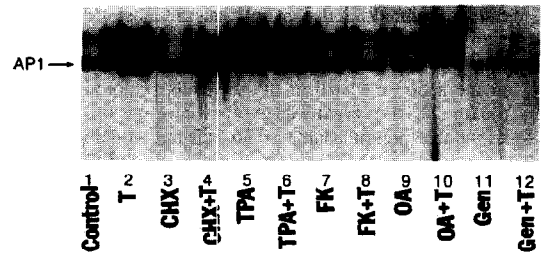

Fig. 5. Autoradiograph representation of the electrophoretic mobility shift (gel mobility shift) assay measuring the effect of TCDD on AP-1 binding activity on DNA. TCDD (10 nM) was added to isolated pieces of adipose tissue in a culture medium with or without the diagnostic agent and incubated for 15 min. The extracted nuclear proteins from control and TCDD-treated adipose tissue were used for the shift assay, as described in Materials and Methods. Different diagnostic chemicals were added to the incubated tissue either separately or in combination with TCDD during incubation. Key: lane 1, control; lane 2, TCDD-treated (T): lane 3, cycloheximide-treated (CHX, 500 μM); lane 4, same as 3 plus TCDD; lane 5, TPA (1 μM); lane 6, same as 5 plus TCDD; lane 7, forskolin (FK, 100 μM); lane 8, same as 7 plus TCDD; lane 9, okadaic acid (OA, 1 μM); lane 10, same as 9 plus TCDD; lane 11, genistein (Gen, 15 μM) and lane 12, same as 11 plus TCDD. Oligonucleotide competition experiments (not shown) were also carried out with unlabeled AP-1 oligonucleotide (20×) using the same samples. The results showed that the lower band (shown by the arrow) represents a more specific binding than the upper band. The data presented are from one of three experiments and were reproducible and consistent.

parent that regulation of these nuclear transcription factors by different mitogenic signals or chemical insults could initially occur through phosphorylation-dephosphorylation [13]. Based on the similarity of action of TPA and TCDD on immediate early gene expressions [39], the possibility of protein phosphorylation processes playing the major role in the action of TCDD should be considered to be very high.

Another key finding in the current work is the stimulation of nuclear MAP2 kinase by TCDD after 15 min of treatment (Table 2). MAP kinase appears to be the central component of many different signal transduction pathways. The activation of MAP kinases has been observed during growth factor stimulation of DNA synthesis and during cell differentiation [40]. This, along with our current data (Table 2) and our previous finding [41] that TCDD caused a rise in *c-Ras* activities in adipose tissue, suggests that an important action of TCDD is to stimulate the growth factor signal transduction pathway.

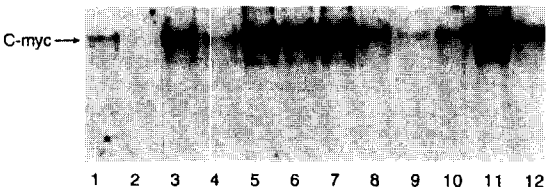

Fig. 6. Autoradiograph representing an EMSA of the c-Myc binding to ^{32}P-labeled c-Myc response element DNA probe. Exactly the same samples as those described in Fig. 5 were tested in the same order against this probe (i.e. the same lane designations). The data presented are from one of three experiments and were reproducible and consistent.

The possibility that TCDD could directly interact with protein kinases is unlikely, based on the observation that TCDD showed almost no effect on isolated microsomal and plasma membrane or nuclear protein kinases [25, 38, 42]. In addition, the observed effect of TCDD on protein phosphorylation activities was more pronounced in a TCDD-responsive strain, C57, than that observed in a less TCDD-responsive strain, DBA [25]. Together, these data strongly support the idea that most of the actions of TCDD, if not all of them, are mediated through the Ah-receptor [38].

Finally, we would like to point out an intriguing observation that in this issue the change in overall protein phosphorylation appeared to be inversely correlated with that of extranuclear fractions (i.e. cytosolic and microsomal). The simplest way to explain the phenomenon is to propose signal-induced translocation of a protein kinase or kinases across the nuclear membrane. However, in terms of total quantity, much higher levels of protein phosphorylation activities are increased in cytosol than those lost from the nucleus. Furthermore, many kinases and phosphatases are not transported freely across the nuclear membrane. Thus, it appears difficult to support such a simplistic idea, although we cannot entirely eliminate the possibility that some kinases or phosphatases are translocated as a result of TCDD signals.

In conclusion, we have firmly established that TCDD at toxicologically relevant doses causes a significant reduction in some nuclear protein phosphorylation in guinea pig adipose tissue. Such an effect of TCDD occurred in parallel with a rise in overall protein phosphorylation at extranuclear locations. In view of the increasing awareness of the functional importance of phosphorylation of nuclear transcription factors [13, 30], this hitherto unsuspected action of TCDD must be considered as a pivotal observation that might provide a clue to solving the puzzle of the molecular mechanism of action of dioxin-type chemicals.

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