Biochemical Pharmacology, Vol. 59, pp. 741–751, 2000.

ISSN 0006-2952/00/$–see front matter
PII S0006-2952(99)00387-1

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD)-Induced Changes in Activities of Nuclear Protein Kinases and Phosphatases Affecting DNA Binding Activity of c-Myc and AP-1 in the Livers of Guinea Pigs

*Hitoshi Ashida, Scott Nagy and Fumio Matsumura**

Department of Environmental Toxicology and Institute of Toxicology and Environmental Health, University of California, Davis, CA 95616, U.S.A.

ABSTRACT. To study the effect of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on nuclear protein phosphorylation activities, male guinea pigs were treated *in vivo* with a single 1 μg/kg i.p. injection of TCDD, and the state of protein kinases and phosphatases in the nuclei of the hepatocytes was examined after 1, 10, and 40 days. TCDD was found to cause a rise in nuclear protein tyrosine kinase on day 1, and to a lesser extent on day 10, but this effect diminished almost completely on day 40. TCDD also caused a reduction in nuclear casein kinase II (CKII) activity at all time points. To study the biochemical events taking place at the early stage of the action of TCDD, a short-term *in vitro* model system was established using explant liver tissues maintained in tissue culture medium. It was found that TCDD caused a rapid reduction of the activity of nuclear CKII with an accompanying increase in the cytosol. Such changes in protein phosphorylation activities were also accompanied by an increase in the DNA binding activity of activator protein 1 (AP-1). The effect of TCDD on nuclear proteins binding to the c-Myc response element DNA was, on the other hand, biphasic: an initial increase of protein binding to the c-Myc response element was followed by suppression. To test the hypothesis that some of the above changes were caused by TCDD-induced changes in protein kinase activity, nuclear proteins isolated from hepatocytes of *in vivo* treated guinea pigs were incubated with exogenously added Mg^{2+} and ATP under cell-free conditions. The results showed that this *in vitro* phosphorylation treatment exacerbated this tendency of increased AP-1 and decreased c-Myc binding to their respective response element DNAs, indicating that kinases and phosphatases present in the isolated nuclear protein preparation were active and capable of modifying protein binding to DNA. Such effects of Mg^{2+} and ATP on AP-1 were blocked by heparin, indicating that CKII plays an important role in transducing the signal of TCDD into the nucleus. BIOCHEM PHARMACOL **59**;7:741–751, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. TCDD; casein kinase II; nuclear transcription factors; guinea pig.

TCDD† is the most potent congener of a family of dioxin-type chemicals that includes many polyhalogenated dioxins, dibenzofurans, and biphenyls [1]. These chemicals are known to cause a wide variety of effects, including carcinogenesis, teratogenesis, immunosuppression, and altered cell proliferation and differentiation. Most of these effects have been shown to be mediated through a cytosolic receptor known as the AhR [2–4]. TCDD has been found to cause a variety of toxic effects, which vary from species to species, between the sexes, and among developmental stages and ages [5, 6]. Because of these variations in symptoms, it has been difficult to find a common biochemical mechanism that could explain all of the actions of TCDD.

One important aspect of the action of TCDD is the alteration of cellular growth and differentiation programs [1–6]. These effects of TCDD are also known to be mediated through the AhR. TCDD has also been shown to cause activation of cAMP-dependent and -independent protein kinases [7], activation of the tyrosine kinase $pp60^{src}$ [8], and increased titers and GTP binding activity of Ras [9–11]. Thus, it has been suggested that TCDD causes activation in the growth factor signal transduction pathway (sometimes referred to as the Ras pathway) [11]. Growth factor signals are known to be transmitted to the nucleus, and, therefore, the assumption made by a few scientists has been that TCDD alters this transmission through modulation of protein phosphorylation activities [11]. For instance, TCDD has been shown to cause a rise in phosphorylation of

* Corresponding author: Dr. Fumio Matsumura, Department of Environmental Toxicology, University of California, Davis, CA 95616. Tel. (530) 752-2769; FAX (530) 752-5300; E-mail: fmatsumura@ucdavis.edu

† *Abbreviations*: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; AhR, aryl hydrocarbon receptor; AP-1, activator protein 1; cAMP, cyclic AMP; EMSA, electrophoretic gel mobility shift assay; DTT, dithiothreitol; CKII, casein kinase II; MAP kinase, mitogen-activated protein kinase; DMEM, Dulbecco's modified Eagle's medium; TIU, trypsin inhibitor unit; and TCA, 1,1,1-trichloroacetic acid.

Received 11 November 1998; accepted 16 August 1999.

$p34^{cdc2}$ kinase [12], an indispensable regulator of the mitotic cell cycle [13, 14]. It is noteworthy that the activation of c-Src is associated intimately with cdc2 activation and mitosis [15].

One way that the growth factor signal transduction pathways induce mitogenic responses is by directly activating "primary response genes" through protein kinase cascades [16]. Activation of these cascades results in changes in the phosphorylation status of nuclear transcription factors such as Jun, Fos, Myc, CREB, and NF-κB [17]. Some of these proteins are phosphorylated in response to external stimuli, such as those induced by growth factors, inflammatory cytokines, and hormones. Others are phosphorylated cyclically in response to the timing mechanism of the cell cycle [18–21]. It is also well known that these changes in the phosphorylation status of nuclear transcription factors alter their DNA binding activity [14, 22].

Recently, by using electrophoretic gel mobility shift assays, we have shown that TCDD administered at 1 μg/kg i.p. *in vivo* causes changes in the binding activity of nuclear transcription factors to specific ^{32}P-labeled DNA probes over the long term [23]. Nuclear protein binding to AP-1- and dioxin-response elements was found to be stimulated by TCDD from day 1 to day 28 post-treatment. TCDD also reduced protein binding to c-Myc and Sp-1 response elements at all time points (1–40 days). The early effects of TCDD are considered to be the result of phosphorylation of the transcription factors themselves. Indeed, Enan and Matsumura [24] have shown, using isolated guinea pig adipose tissue in culture medium, that TCDD regulates the DNA binding activity of AP-1 and c-Myc primarily through modulating their state of phosphorylation *in vitro*. They concluded that the effects are likely due to altered protein kinase and phosphatase activities. They also demonstrated that the initial effect takes place very rapidly (1–10 min) and is dependent on the AhR.

In the current work, we have examined whether TCDD-induced changes in the DNA binding activity of AP-1 and c-Myc are also mediated by protein phosphorylation, at least in part. Because many of the transcription factors previously found to be affected by TCDD are phosphorylated by CKII, we chose to study this phenomenon in detail.

MATERIALS AND METHODS

Materials

TCDD was a gift from the Dow Chemical Co. with a purity higher than 99.99%. [γ-^{32}P]ATP (~3000 mCi/mmol) was obtained from Amersham. Dephosphorylated α-casein, histone (type III-S), the $pp60^{src}$ specific peptide substrate (RR-SRC), aprotinin, and leupeptin were purchased from the Sigma Chemical Co. The specific peptide substrate for CKII (RRREEETEEE) was obtained from Peninsula Laboratories, Inc. The double-stranded oligonucleotide containing the consensus Myc–Max response element (5′-GGA AGC AGA CCA CGT GGT CTG CTT CC-3′) and the AP-1 response element (5′-CTA GTG ATG AGT CAG CCG GAT C-3′) were purchased from Santa Cruz Biotechnology, Inc. A polyclonal antibody to the α and α′ subunits of CKII was purchased from Upstate Biotechnology, Inc., and a monoclonal antibody to c-Myc was purchased from Oncogene Science Inc. Polyclonal antibodies to p-53 and c-Jun were purchased from Santa Cruz Biotechnology, Inc. DMEM (23800–022) was obtained from GIBCO BRL Life Technologies, Inc. All other chemicals were of the highest grade available from commercial sources.

General Experimental Design

The effects of TCDD on nuclear protein phosphorylation in the liver were studied in both *in vivo* and *in vitro* experiments. For the *in vivo* study, animal treatment was carried out by the injection of TCDD into three male guinea pigs in each experimental group as described in detail in the "Treatment of Animals" section. Nuclear protein extracts were prepared and used in the following experiments: The activities of major classes of kinases and phosphatases were surveyed using extracts from animals 1 day after treatment. The status of total phosphorylation was examined in the nuclear protein fractions from days 1, 10, and 40. At the same time points, the activities of protein tyrosine kinases, phosphatases, and CKII were measured using corresponding suitable substrates. Finally, we addressed the effects of exogenously added inhibitors and stimulators of protein phosphorylation on the DNA binding activity of c-Myc and AP-1.

To study the *in vitro* action of TCDD at an early stage, we employed liver explant culture because explanted tissue consists of cells from the whole liver, i.e. not only parenchymal hepatocytes but also non-parenchymal cells such as Kupffer cells and Ito cells. The culture was performed in triplicate, and the extracted nuclear protein from the cultures was used for measurements of CKII activity, the levels of this enzyme protein and related substrates by western blotting analysis, and the DNA binding activity of c-Myc and AP-1.

Treatment of Animals

Four- to-six-week-old (200–225 g), male English short-hair guinea pigs (*Avia porcellus*) were housed in suspended steel cages, provided with food and water *ad lib.*, and maintained in a controlled room (temperature 22–23°, humidity 70%, 12 hr light/12 hr dark cycle). Animals were divided at random into two groups of nine guinea pigs each. One group was treated with TCDD by a single i.p. injection (1 μg/kg body weight) in a mixture of corn oil:acetone (9:1), and the controls received an equal volume (0.1 mL) of the vehicle only. Three animals in each experimental group were decapitated at specific post-treatment intervals (1, 10, and 40 days), between 9:00 and 10:00 a.m. to avoid possible effects due to diurnal variation. The liver was taken out

promptly, perfused with a 1.15% KCl solution, frozen in liquid nitrogen, and stored at -80° until used.

In Vitro *Treatment of Isolated Liver Tissue*

For the *in vitro* TCDD treatment experiments, 1 g of fresh liver from untreated guinea pigs was incubated with 10^{-8} M TCDD or solvent (10 μL of 1,4-dioxane) in 5 mL of DMEM (containing 13.3 mM glucose) in a 5% CO_2, 37° environment. After incubation for 15, 30, 60, or 120 min, the tissue was washed three times with 5 mL of fresh medium and once with 5 mL of homogenizing buffer (10 mM HEPES, pH 7.4, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 7.8 mTIU/mL of aprotinin, and 2.25 μg/mL of leupeptin). The tissue was used immediately for the preparation of the nuclear protein fraction.

Preparation of Hepatic Subcellular Fractions

The nuclear protein fraction of each liver sample from the *in vivo* or the *in vitro* explant tissue treatments was prepared separately as described previously [23]. Briefly, liver (1 g) was homogenized in 3 vol. (w/v) of the homogenizing buffer with six strokes of a Teflon/glass homogenizer. The homogenates were centrifuged at 1000 *g* for 10 min at 4° in a Sorvall™ SS34 rotor to pellet crude nuclei. The pellet was washed three times with homogenizing buffer using the same centrifuge conditions. Next, the pellet was suspended in 1.4 mL of extraction buffer (20 mM HEPES, pH 7.4, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM DTT, 7.8 mTIU/mL of aprotinin, and 2.25 μg/mL of leupeptin) and homogenized with ten strokes of a glass/glass homogenizer. The homogenate was transferred to an Eppendorf tube and kept on ice for 30 min. After shaking for a further 30 min, the homogenate was centrifuged at 16,000 *g* for 20 min at 4°. The clear supernatant obtained was referred to as the nuclear protein fraction. The protein concentration in the nuclear protein fraction was determined by the method of Lowry *et al.* [25], using bovine serum albumin as a standard protein. Nuclear fraction was frozen at −80° until used.

Total Phosphorylation Assays

For total protein phosphorylation, the reaction mixtures (final volume 60 μL) contained 50 μg of nuclear protein in 50 mM HEPES, pH 7.4, containing 10 mM $MgCl_2$ with or without 0.4 μM heparin (inhibitor of CKII), or in the same buffer containing 10 mM $MnCl_2$ and 10 μM Na_3VO_4 with or without 15 μM genistein (inhibitor of protein tyrosine kinase). The reaction was started by the addition of 0.5 μCi [γ-^{32}P]ATP (1 μM) and was terminated 2 min after incubation at 37° by the addition of 20 μL of 4x SDS–treatment buffer. The mixture was heated at 90° for 3 min, and the entire volume was loaded on a 10% SDS–polyacrylamide gel. After electrophoresis, the gels were stained, destained, dried, and exposed to x-ray film. Total phosphorylation was measured by counting the radioactivity in each lane using an AMBIS Radioanalytical Imaging System (AMBIS Systems Inc.).

Measurement of Nuclear Protein Kinase and Phosphatase Activities

To survey the types of nuclear protein kinases and phosphatases affected by *in vivo* treatment with TCDD, an aliquot (20 μg) of nuclear protein was incubated with 40 μg of histone as a substrate in 40 μL of reaction buffer (50 mM HEPES, pH 7.5, 10 mM $MgCl_2$) with suitable inhibitors and activators as follows: 15 μM genistein was used as a specific inhibitor of protein tyrosine kinase; 10 μM Na_3VO_4 was used as a specific inhibitor of tyrosine phosphatase; 0.1 μM okadaic acid was used as a specific inhibitor of protein phosphatases 1A and 2A; 0.4 μM heparin was used as a specific inhibitor of CKII; 1 μM cAMP was used as an activator of protein kinase A; 1.83 mM $CaCl_2$ was used as an activator of calcium-dependent protein kinases; and 10 U calmodulin and 0.3 mM $CaCl_2$ were used as activators of calmodulin-dependent kinases. For specific activities of protein tyrosine kinase and phosphatase, the reactions also were carried out in a final volume of 40 μL containing 50 mM HEPES, pH 7.5, 10 mM $MnCl_2$, and 500 μM specific substrate peptide, RR-SRC, with a combination of 15 μM genistein and 10 μM Na_3VO_4. After preincubation for 2 min at 37°, the reaction was started by the addition of 1 μM [γ-^{32}P]ATP (0.5 μCi) for 60 sec and stopped by the addition of 4 μL of 20% TCA. A half-volume of the reaction mixture (22 μL containing 10 μg protein) was spotted onto a 2-cm^2 piece of phosphocellulose paper, which was washed three times for 5 min in 3 mL of 85 mM H_3PO_4, dried at room temperature, put in a vial with scintillation fluid, and assayed for radioactivity.

Measurement of CKII Activity

CKII activity was measured as described by Enan and Matsumura [24] with some modifications. Briefly, 20 μg of nuclear protein was incubated with 80 μg of dephosphorylated casein in 100 μL of reaction buffer (50 mM HEPES, pH 7.6, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT) containing various amounts of heparin. After 2 min of incubation, the reaction was initiated by adding 0.2 μCi of [γ-^{32}P]ATP (final concentration 1 μM) and terminated by the addition of 0.5 mL of 20% TCA. After keeping it on ice for 5 min, 0.5 mL of the reaction mixture was filtered through a 0.45-μm filter (Millipore, HAWP 025 00), and the filter paper was washed six times with 1 mL of 20% TCA. After drying, the filter papers were counted in 4 mL of liquid scintillation fluid.

CKII activity was also measured using its specific peptide substrate (RRREEETEEE [26]) with the phosphocellulose paper methods described above. Reactions were carried out in a final volume of 40 μL containing 50 mM 3-(*N*-

morpholino)propanesulfonic acid (MOPS), pH 7.0, 10 mM $MgCl_2$, 1 mM DTT, 150 mM NaCl, 0.5 mM peptide substrate, 20 μg of nuclear protein, and 1.0 μCi [γ-^{32}P]ATP (final concentration 1 μM).

EMSA

c-Myc and AP-1 response elements were individually 5′-end-labeled using T4-polynucleotide kinase and [γ-^{32}P]ATP [27] and used as probes in the binding assay. The binding reaction mixture (final volume 25 μL) contained 20 μg of nuclear protein, 1 μg of poly dI-dC, and [γ-^{32}P]-labeled probe (100,000 cpm, 0.2 to 0.4 ng) in the binding buffer (25 mM HEPES, pH 7.5, 1 mM EDTA, 10% glycerol, and 1 mM DTT). The reaction was carried out in two stages. All components except the probe were mixed together and preincubated at 4° for 15 min to allow nonspecific DNA binding to proteins. Following the addition of the ^{32}P-labeled probe, incubation was continued for an additional 20 min at room temperature. The reactions were terminated by loading samples on a 6% (29:1 cross-linking ratio of acrylamide and bisacrylamide) non-stacking native polyacrylamide gel containing 0.25x TBE (25 mM Tris, 22.5 mM borate, and 0.25 mM EDTA). In each set of experiments, a competition assay was performed using a 100-fold excess of the unlabeled probe to obtain nonspecific binding activity. The gel was subjected to electrophoresis in 0.25x TBE at 180 V for 30 min before loading and for 70 min after loading. After electrophoresis, gels were dried and exposed to x-ray film. The level of radioactivity at the binding sites was measured by the AMBIS Radioanalytical Imaging System.

When the effects of exogenously added inhibitors and stimulators of protein phosphorylation on DNA binding activity were determined, nuclear protein (100 μg) was incubated in combination with the following chemicals: $MgCl_2$ (10 mM), ATP (1 μM), okadaic acid (0.1 μM), Na_3VO_4 (10 μM), genistein (15 μM), heparin (0.4 μM), and EDTA (10 mM). After incubation for 5 min at 37°, an aliquot of each mixture containing 20 μg protein was subjected to EMSA.

Western Blot Analysis

Nuclear proteins, 20 μg for analysis of CKII and c-Jun blots and 80 μg for c-Myc and p53 blots, were resolved by 10% SDS–PAGE and transferred to nitrocellulose membranes at 60 V for 90 min. The blots were blocked overnight in TBS–Tween (10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween 20) containing 5% nonfat dry milk, and then incubated with primary antibodies according to the manufacturer's directions. The membranes were washed with TBS–Tween, and the blots were incubated with a horseradish peroxidase-conjugated secondary antibody (Amersham). After washing with TBS–Tween, the immunocomplexes were visualized with the ECL antibody detection system (Amersham). The intensities of the immunocomplexes were quantified by the AMBIS system.

TABLE 1. *In vivo* effect of TCDD on nuclear protein phosphorylation in the presence of inhibitor and activator for various phosphatases and kinases

Treatment	Phosphorylation on histone (fmol/min/μg protein)	
	Control	TCDD
(a) None	30.2 ± 0.2	24.8 ± 0.7*
(b) Vanadate, 10 μM	38.3 ± 1.2†	30.6 ± 0.9*†
(c) Genistein, 15 μM	28.6 ± 0.2†	20.5 ± 0.5*†
(d) Okadaic acid, 100 nM	34.5 ± 0.8†	28.8 ± 1.2*†
(e) Heparin, 400 nM	23.8 ± 0.5†	20.7 ± 1.6†
(f) Cyclic AMP, 1 μM	30.0 ± 0.8	23.6 ± 0.8*
(g) Calcium, 1.83 mM	33.9 ± 0.6	24.9 ± 0.8*
(h) Calcium, 0.3 mM + calmodulin, 10 U	31.5 ± 0.8	24.9 ± 1.0*

Hepatic nuclear proteins were prepared from TCDD-treated (1 μg/kg, i.p.) or control guinea pigs 1 day after treatment. The nuclear protein phosphorylation was measured using histone as a general substrate as described in Materials and Methods. Data are means ± SD from three animals.

*Significantly different from the corresponding control values ($P \leq 0.05$), when the TCDD effect was analyzed by Student's *t*-test.

†Signifciantly different from the no treatment values ($P \leq 0.01$), when the treated chemical effect was analyzed by two-way fractional ANOVA and multiple comparison tests using Scheffe's method as a post-hoc test.

Statistical Analysis

Statistical analysis was performed by computer using StatView (ver. 4.0J). Student's *t*-test was used to determine the statistical significance of differences between the TCDD-treated values and the control values. Multiple comparisons were carried out by two-way fractional ANOVA with Scheffe's method as a post-hoc test. In both statistical analyses, $P \leq 0.05$ was chosen as the level of significance.

RESULTS

In the first series of experiments, we surveyed the activity of the major classes of kinases and phosphatases in isolated hepatic nucleus preparations from guinea pigs treated *in vivo* with 1 μg/kg of TCDD for 24 hr. The data summarized in Table 1 demonstrate that the level of phosphorylation in the nuclear fraction was lower in the preparations from treated animals than in those from controls in all cases. This result was consistent with our previous report [24], which showed that *in vivo* treatment with TCDD reduces overall phosphorylation in nuclei from guinea pig adipose tissue, whereas the same treatment causes an increase in the level of phosphorylation in the cytosol and particulate fractions. Exogenous addition of vanadate, genistein, okadaic acid, or heparin to nuclear extracts modulated the phosphorylation status. Vanadate and okadaic acid, which are specific inhibitors of protein tyrosine phosphatase and protein phosphatase 1A and 2A, respectively, increased the level of phosphorylation. On the other hand, genistein and

heparin, which are inhibitors of protein tyrosine kinases and heparin-sensitive kinases, respectively, suppressed the phosphorylation level. These results indicated that active phosphatases and kinases were present in the prepared nuclear fraction. To make clear the effect of TCDD on protein phosphorylation, the absolute difference was calculated between the values in the presence and absence of each chemical: TCDD reduced the level of vanadate-sensitive phosphatase activity [i.e. (b) − (a) = 5.8 fmol/min/μg protein for TCDD-treated and 8.1 fmol/min/μg protein for control]. In contrast, the level of okadaic acid-sensitive phosphatase activity remained unchanged. These data indicated that TCDD lowered the levels of protein tyrosine phosphatases but not those of protein phosphatase 1A and 2A. TCDD increased the activity of genistein-sensitive tyrosine kinases [i.e. (a) − (c) = 4.3 fmol/min/μg protein for TCDD-treated and 1.6 fmol/min/μg protein for control] despite the fact that TCDD decreased overall phosphorylation. CKII, which is a heparin-sensitive kinase, is a candidate kinase for the alteration of the DNA binding activities of AP-1 and c-Myc induced in adipose tissue by TCDD [24]. In the current study, heparin decreased kinase activity in TCDD-treated animals [i.e. (a) − (e) = 4.1 fmol/min/μg protein for TCDD-treated and 6.4 fmol/min/μg protein for control]. Thus, these enzymes could play important roles in affecting the DNA binding activities of transcription factors via changes in their phosphorylation status. The addition of cyclic AMP, calcium, and calcium plus calmodulin to the nuclear preparation did not show any chemical effect on phosphorylation, indicating that the activities of cyclic AMP-, calcium-, and calmodulin-dependent kinases are not prominent in the preparation.

One of the main questions raised by the above experiment was the timing of changes in the activity of protein tyrosine kinases and phosphatases following TCDD treatment. To obtain an answer for this question, we analyzed total phosphorylation activity by SDS–PAGE in nuclear extracts from liver following the *in vivo* administration of TCDD on days 1, 10, and 40. As expected, the genistein-sensitive protein kinase activities increased 1 day after treatment (Fig. 1A). However, this increase appeared to be transient, since the level of phosphorylation diminished by days 10 and 40. These data suggested that protein tyrosine kinases in the nucleus may be activated initially by TCDD and subsequently down-regulated. In contrast, TCDD reduced the levels of heparin-sensitive phosphorylation activities at all time points tested, indicating that probably CKII is down-regulated by TCDD (Fig. 1B).

Figure 2 shows the time-course effect of TCDD on protein phosphorylation when histone was used as a substrate in the hepatic nuclear fraction. TCDD caused a significant increase in the level of genistein-sensitive tyrosine kinase activities under these conditions both in the absence (panel A) and in the presence of vanadate (panel B) on day 1, and this increase lasted through day 10. By day 40, however, there was a decrease in genistein-sensitive

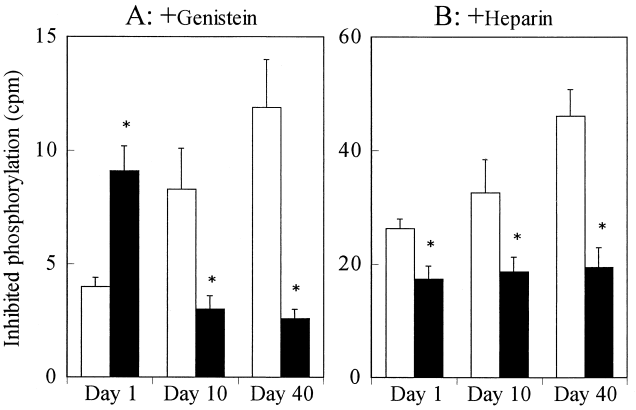

FIG. 1. Effect of TCDD administered *in vivo* on the levels of nuclear protein phosphorylation affected by genistein- and heparin-sensitive kinases. Guinea pigs were given a single i.p. injection of TCDD (1 μg/kg) or vehicle alone [0.1 mL of a corn oil:acetone mixture (9:1)] and killed on day 1, 10, or 40. Hepatic nuclear proteins were prepared as described in Materials and Methods. (A) Nuclear proteins were phosphorylated using 0.5 μCi [γ-^{32}P]ATP (1 μM) in a reaction buffer (50 mM HEPES, pH 7.5, and 40 μg histone as a substrate in a 40-μL reaction mixture) containing 10 mM $MnCl_2$ and 10 μM Na_3VO_4 in the presence or absence of 15 μM genistein. (B) Nuclear proteins were phosphorylated using 0.5 μCi [γ-^{32}P]ATP (1 μM) in the same buffer containing 10 mM $MgCl_2$ in the presence or absence of 0.4 μM heparin. The phosphorylated products were analyzed using SDS–PAGE and quantitated by the AMBIS radioimaging system. Data are represented by subtraction of the values in the presence of genistein or heparin from the values in the presence of inhibitors. Open bars, control; and solid bars, TCDD-treated. Values are means ± SD from three animals. Key: (*) significantly different from control ($P < 0.05$, Student's *t*-test).

tyrosine kinase activity as compared with control, perhaps demonstrating a negative feedback mechanism. TCDD had an opposite effect on vanadate-sensitive tyrosine phosphatases. Panels C and D of Fig. 2 show that TCDD lowered the vanadate-sensitive phosphatase activity on day 1, and that the decreased phosphatase activity in TCDD-treated nuclei gradually increased after treatment. There was no difference in the phosphatase activity between control and treated samples on day 10, but by day 40, the level was significantly higher in the treated samples than in the control. It was noteworthy that these actions of TCDD on tyrosine kinases (Fig. 3A) and phosphatases (Fig. 3B) were also observed using the artificial peptide substrate RR-SRC, which is known to be favored by Src-type protein tyrosine kinases. Changes in the activities of Src-type tyrosine kinases by TCDD were shown to be more pronounced as compared with the data in Fig. 2 on both days 1 and 40. The activity of tyrosine phosphatase towards RR-SRC was found to be similar to that towards histone on days 1 and 40.

The activity of CKII was measured next, using three different substrates, casein (Fig. 4A), histone (Fig. 4B), and the specific peptide substrate RRREEETEEE for CKII (Fig. 4C), throughout the same time period. TCDD was found to

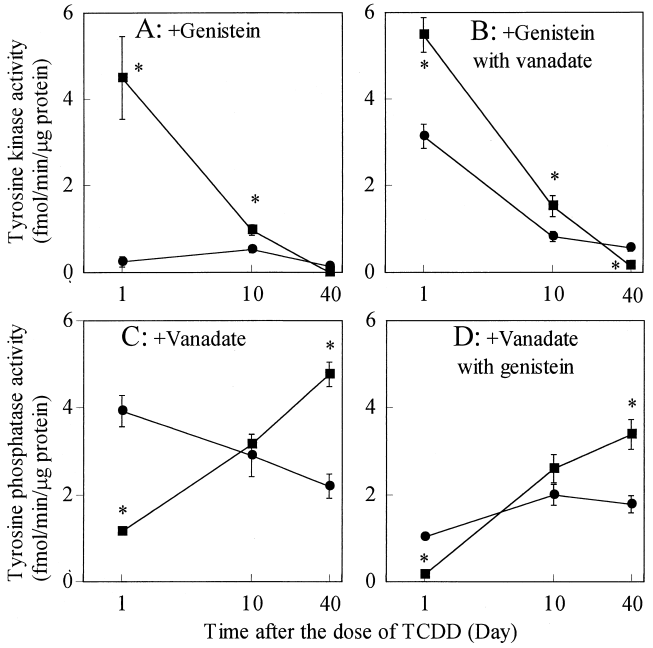

FIG. 2. Time–course effect of TCDD administered *in vivo* on the activity of protein phosphorylation in hepatic nuclei depending on the levels of genistein-sensitive kinases and vanadate-sensitive phosphatases assessed by using histone as a substrate. Hepatic nuclear proteins were prepared from TCDD-treated and control guinea pigs as described in the legend of Fig. 1. The activity of protein phosphorylation in the nuclear protein preparation was measured in a reaction buffer (50 mM HEPES, pH 7.5, 10 mM $MnCl_2$, and 40 μg histone as a substrate in a 40-μL reaction mixture) with a combination of 15 μM genistein and 10 μM Na_3VO_4, using phosphocellulose paper. The level of protein tyrosine kinase activities was calculated from the ^{32}P-phosphorylation inhibited by genistein in the absence (A) or presence (B) of vanadate, whereas that of phosphatase activities was calculated from the ^{32}P-phosphorylation inhibited by vanadate in the absence (C) or presence (D) of genistein. Circles, control; squares, TCDD-treated. Values are means ± SD from three animals. Key: (*) significantly different from control ($P \leq 0.05$, Student's *t*-test).

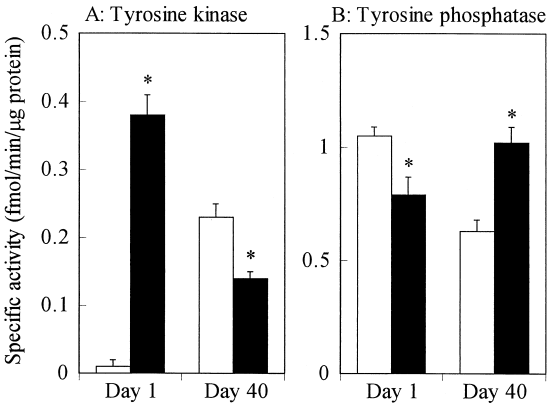

FIG. 3. Time–course effect of TCDD administered *in vivo* on the activities of genistein-sensitive protein tyrosine kinases and vanadate-sensitive phosphatases in hepatic nuclei assessed by using RR-SRC as a specific substrate. Hepatic nuclear proteins were prepared from TCDD-treated and control guinea pigs as described in the legend of Fig. 1. The activity of protein phosphorylation in nuclear protein preparations was measured in a reaction buffer (50 mM HEPES, pH 7.5, 10 mM $MnCl_2$, and 500 μM specific substrate peptide RR-SRC in a 40-μL reaction mixture) with a combination of 15 μM genistein and 10 μM Na_3VO_4, using phosphocellulose paper. The activity of protein tyrosine kinases (A) was calculated from the ^{32}P-phosphorylation inhibited by genistein in the presence of vanadate, while that of phosphatases (B) was calculated from the ^{32}P-phosphorylation inhibited by vanadate in the presence of genistein. Open bars, control; and solid bars, TCDD-treated. Values are means ± SD from three animals. Key: (*) significantly different from control ($P \leq 0.05$, Student's *t*-test).

suppress CKII activity *in vivo* in the nucleus at all time points tested, regardless of the substrate chosen. We also measured cytosolic CKII and observed that TCDD increased its activity slightly on day 10, followed by a significant decrease by day 40 (data not shown).

In the next series of experiments, to determine whether the biochemical events of TCDD action, such as down-regulation of CKII, take place at an early stage, we studied the effect of TCDD (10 nM) on CKII activity *in vitro*, using isolated explants of hepatic tissue in culture medium. This method allowed us to study the effect of TCDD on liver cells without disrupting their cellular organization, unlike primary hepatocyte cultures. To avoid deterioration of tissues we adopted only short exposure periods. By 15 min, there was a significant reduction in CKII phosphorylation activity on casein (Fig. 5A), histone (Fig. 5B), and the specific peptide substrate (Fig. 5C). The inhibition was observed at all time points tested in a time-dependent manner, and finally became approximately 50% for all three substrates by 120 min. To determine whether the differences observed in CKII activity between these preparations were due to the quantitative difference in CKII

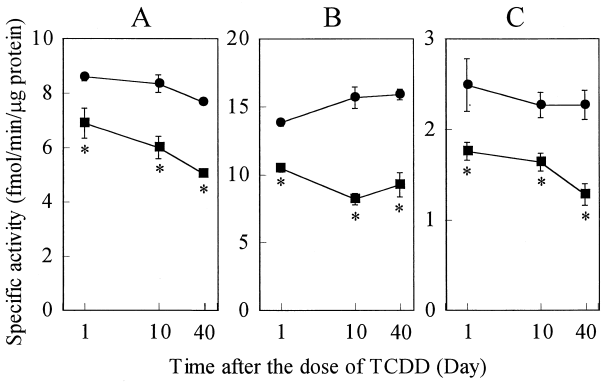

FIG. 4. Time–course effect of TCDD administered *in vivo* on CKII activity in hepatic nuclear proteins as measured with three different substrates. Hepatic nuclear proteins were prepared from TCDD-treated and control guinea pigs as described in the legend of Fig. 1. CKII activity was measured in hepatic nuclear protein preparation using three kinds of substrates: casein (A), histone (B), or the specific peptide RRREEETEEE (C) as described in Materials and Methods. Circles, control; and squares, TCDD-treated. Values are means ± SD from three animals. Key: (*) significantly different from control ($P \leq 0.05$, Student's *t*-test).

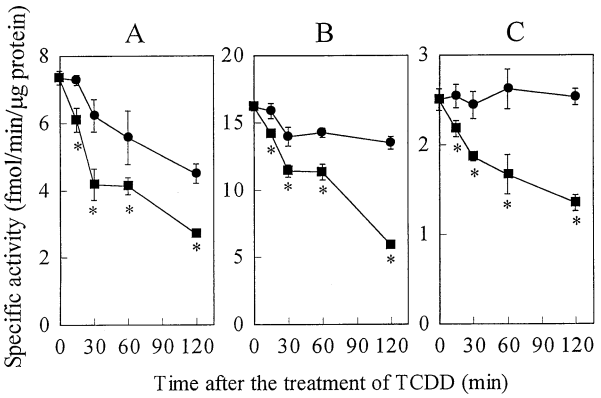

FIG. 5. Time–course effect of *in vitro* treatment with TCDD on CKII activity in nuclear protein preparations from explant-cultured liver tissue. Liver tissue (1 g) from untreated guinea pigs was isolated and incubated with 10 nM TCDD or solvent (10 μL of 1,4-dioxane) in 5 mL of DMEM for 15, 30, 60, and 120 min. At each time point, nuclear proteins were prepared from the tissue, and CKII activity was measured using three kinds of substrates: casein (A), histone (B), or the specific peptide RRREEETEEE (C) as described in Materials and Methods. Circles, control; and squares, TCDD-treated. Values are means ± SD from representative triplicates. Key: (*) significantly different from control ($P \leq 0.01$, Student's *t*-test).

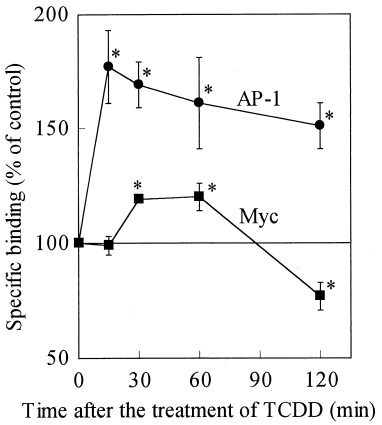

FIG. 6. Effect of *in vitro* treatment with TCDD on DNA binding activity of nuclear proteins to ^{32}P-labeled c-Myc and AP-1 response elements in nuclear protein preparations from explant-cultured liver tissue. Liver tissue (1 g) from untreated guinea pigs was incubated with 10 nM TCDD as described in the legend of Fig. 5. At the time points indicated in the figure, nuclear proteins were prepared from the tissue, and DNA binding activity to ^{32}P-labeled c-Myc and AP-1 response elements was measured by EMSA as described in Materials and Methods. For each probe, specific binding was calculated by subtracting nonspecific binding in the presence of 100-fold excess unlabeled probe. Data are represented as a percent of the specific binding in the control. Values are means ± SD from representative triplicates. Key: (*) significantly different from control ($P \leq 0.01$, Student's *t*-test). The actual values shown in this figure were estimated from the densitometric reading in arbitrary units of the specific band intensities (minus nonspecific background) on the x-ray film from autoradiography. No units are needed.

protein levels, western blot analysis was carried out on nuclear extracts from the *in vitro* treated explant liver tissue. As shown in Table 2, CKII protein levels in treated samples decreased slightly but not significantly by 120 min, and this reduction was not enough to account for the 50% decrease in enzyme activity (Fig. 5). Table 2 also shows the levels of the reported natural substrates of CKII in the nucleus. At the same time point, the protein level of c-Jun in treated samples decreased to 55% of control, although there were no significant effects on the levels of c-Myc and p53. These results indicate that the down-regulation of CKII also was observed in short-term treatments with TCDD *in vitro*, and the reduced activity was not due to the loss of enzyme protein.

Finally, we asked the question whether TCDD could modulate the DNA binding activity of nuclear transcription factors AP-1 and c-Myc by changing their states of protein phosphorylation. We addressed this question using EMSA for AP-1 and c-Myc binding to their corresponding DNA response elements. As shown in Fig. 6, the *in vitro* treatment with TCDD caused an apparent increase in binding to AP-1 response element DNA by 15 through 120 min. DNA binding activity to the c-Myc response element was biphasic. TCDD caused a significant increase in c-Myc binding through 30–60 min, followed by a decrease at 120 min. A similar result was obtained using EMSA in hepatic nuclear extracts from the *in vivo* TCDD-treated animals. TCDD decreased the binding activity of nuclear proteins to the c-Myc response element, whereas it increased binding to the AP-1 response element without preincubation on both days 1 and 40 (Figs. 7 and 8).

Figures 7 and 8 also show the effects of exogenous inhibitors and stimulators of protein phosphorylation on DNA binding activity. With regard to the c-Myc response element, on day 1, the binding activities generally were reduced in the control, whereas they were increased in TCDD-treated nuclear extracts. These results indicate that phosphatases were present predominately in the control, and kinases were activated by TCDD. The addition of both Mg^{2+} and ATP to nuclear extracts increased the DNA binding activity as expected, but these chemicals drowned

TABLE 2. Effect of *in vitro* treatment of TCDD on the levels of CKII, c-Myc, c-Jun, and p53 proteins in nuclear protein extracts from explant-cultured liver of guinea pigs

Protein	Intensity*	
	Control	TCDD (10 nM)
CKII (α and α′ subunits)	26.6 ± 2.1	19.7 ± 2.9
c-Myc	17.7 ± 4.0	20.4 ± 1.5
c-Jun	29.3 ± 0.9	16.1 ± 2.3†
p53	6.4 ± 0.9	4.4 ± 0.8

Liver tissue (1.0 g) from untreated guinea pigs was incubated with 10 nM TCDD or solvent (10 μL 1,4-dioxane) in 5 mL DMEM for 120 min. Nuclear protein was extracted and used for western blotting as described in Materials and Methods. Values are means ± SD of three different experiments.

*Intensity of specific bands of CKII (α and α′ subunits, 40 and 44 kDa, respectively), c-Myc (67 kDa), c-Jun (39 kDa), and p53 (53 kDa) were determined by the AMBIS imaging system.

†Significantly different from control value by Student's *t*-test ($P \leq 0.05$).

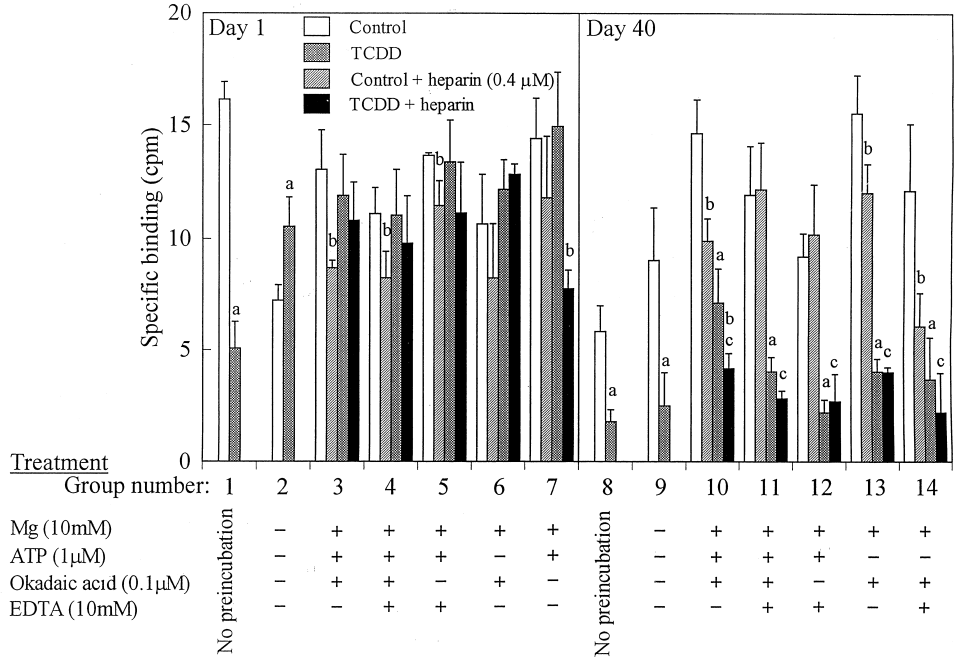

FIG. 7. Effect of cell-free incubation of nuclear protein preparations from a TCDD-treated guinea pig with several phosphorylation modifying agents on DNA binding activity of nuclear protein to ^{32}P-labeled c-Myc response element. Nuclear proteins from the liver of *in vivo* TCDD-treated (1 μg/kg) or control guinea pigs were prepared, and DNA binding activity to ^{32}P-labeled c-Myc response element was measured by EMSA as described in Materials and Methods. Specific binding activity was calculated by subtracting nonspecific binding in the presence of 100-fold excess unlabeled probe. Data are represented as a percent of the specific binding in the control (means ± SD from three animals). Key: "a," significant difference from control ($P \leq 0.05$, i.e. TCDD effect); "b," significant difference from samples without heparin ($P \leq 0.05$, i.e. heparin effect); and "c," significant difference from control with heparin ($P \leq 0.05$, i.e. TCDD effect in the presence of heparin).

out the effect of TCDD (e.g. groups 3–7). Heparin reduced the binding activity in all treatment groups, indicating that heparin-sensitive kinases play an important role in the binding of nuclear protein to the c-Myc response element.

An interesting result was observed when heparin was present with both Mg^{2+} and ATP: the trend of the binding activity was similar to the results of the no incubation group, i.e. TCDD suppressed the binding of the c-Myc

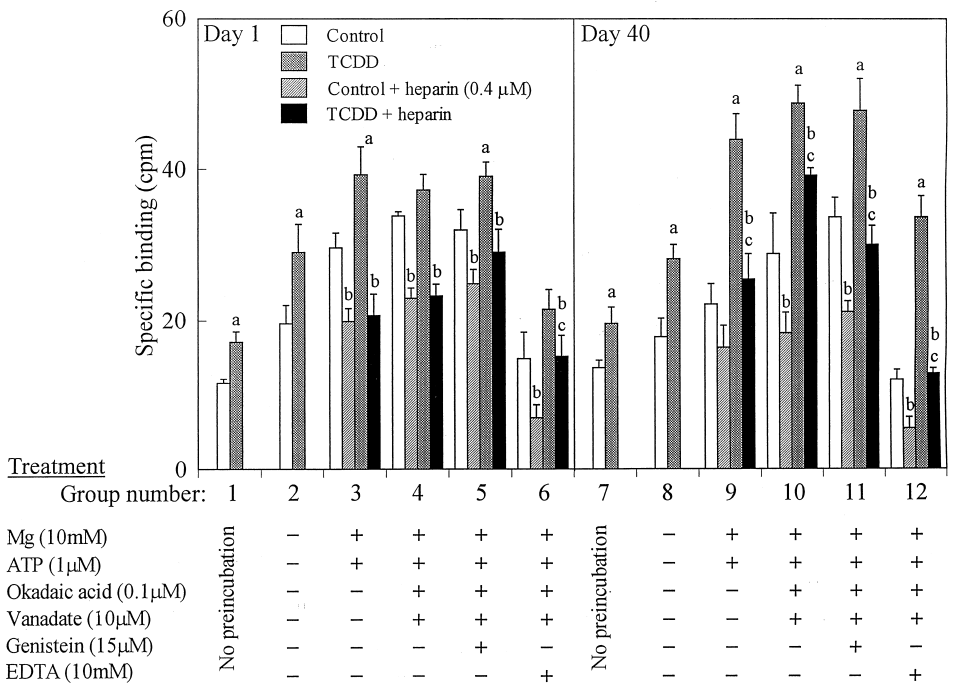

FIG. 8. Effect of cell-free incubation of nuclear protein preparations from a TCDD-treated guinea pig with several phosphorylation modifying agents on DNA binding activity of nuclear protein to ^{32}P-labeled AP-1 response element. All experimental conditions were identical to those described in the legend of Fig. 7 except the use of ^{32}P-labeled AP-1 response element in place of ^{32}P-labeled c-Myc response element. Key: "a," significant difference from control ($P \leq 0.05$, i.e. TCDD effect); "b," significant difference from samples without heparin ($P \leq 0.05$, i.e. heparin effect); and "c," significant difference from control with heparin ($P \leq 0.05$, i.e. TCDD effect in the presence of heparin).

response element (compare group 3 with group 1). However, the addition of okadaic acid restored the suppressive effects of TCDD in the presence of heparin (groups 5 and 6). These results suggest that heparin-sensitive kinases were more active in control extracts than in TCDD-treated extracts when phosphatase activities were blocked by okadaic acid. On day 40, binding of TCDD-treated nuclear extracts to the c-Myc response element was always lower than that of control extracts. The addition of both Mg^{2+} and ATP increased DNA binding activity, especially in control samples (groups 10–12), and this increased effect was suppressed by EDTA, which partially traps Mg^{2+}. An inhibitory effect of heparin towards this increase was observed only in the presence of okadaic acid (group 11), because okadaic acid increased the binding activity in TCDD-treated nuclear proteins. These results suggest that okadaic acid-sensitive phosphatases could modulate the DNA binding activity to the c-Myc response element, cooperating with heparin-sensitive kinases.

Figure 8 shows the DNA binding activity to the AP-1 response element after preincubation with exogenously added chemicals. TCDD caused a significant increase in the binding activity of nuclear proteins in all cases on both days 1 and 40. General trends of the added chemicals were as follows: The addition of both Mg^{2+} and ATP increased DNA binding activity. Okadaic acid, vanadate, and genistein did not affect the pattern of DNA binding. On day 1, heparin obviously abolished the stimulatory effects produced not only by both Mg^{2+} and ATP but also by TCDD, indicating that a role of CKII in nuclear extracts is important for AP-1 binding. On day 40, heparin also showed a significant suppressive effect, but it did not abolish the stimulatory action of TCDD. Thus, contrary to the early stage (i.e. day 1), at the late stage (day 40) other serine/threonine kinases may dominate the AP-1 binding.

DISCUSSION

In the current study, we have established that TCDD, administered *in vivo* to animals or *in vitro* to explant tissue, caused changes in activities of protein kinases and phosphatases in the nuclei of guinea pig hepatocytes. The most prominent kinase affected in this manner was heparin-sensitive CKII, followed by genistein-sensitive protein kinase(s). The enzyme activities of calcium kinases or cAMP-dependent protein kinases were not very high in this preparation. Regarding phosphatases, both okadaic acid-sensitive serine phosphatases (phosphatase 1A and 2A) and vanadate-sensitive phosphatases were recognized in nuclei. The effect of TCDD was expressed more prominently on the latter phosphatases (Table 1).

As for the effect of TCDD, at the early stage of its action (day 1), there was a clear-cut increase in protein tyrosine kinase activity, which was accompanied by down-regulation of protein tyrosine phosphatase activities. Interestingly, at a later stage (day 40), the above tendency was reversed, i.e. down-regulation of kinase and up-regulation of phosphatase. In contrast, the activity of CKII in the nuclear preparations from TCDD-treated animals was always down-regulated, confirming the previous finding of Enan and Matsumura [24]. Such a tendency was very consistent in all test results obtained with three different substrates, and in different preparations from 1- and 40-day exposure samples.

The key question we must ask now is whether such TCDD-induced changes in nuclear protein phosphorylation activities actually cause significant functional modifications in the behavior of key nuclear transcription factors. For this reason, we have chosen two well-studied transcription factors whose DNA binding activities are well known to be influenced by protein phosphorylation [16, 17]. The approach we adopted was EMSA after incubation of isolated nuclear proteins with ATP plus Mg^{2+}, okadaic acid, VO_4^{3-} plus genistein, and/or EDTA. All of these agents are well known to specifically alter the state of phosphorylation, except for EDTA, which could chelate any divalent cations including essential ions such as Zn^{2+}, and, therefore, its action may not be so specific. Of these, protein binding to [^{32}P]c-Myc response element was affected more by the presence of okadaic acid, indicating that protein serine/threonine phosphatases, likely those belonging to the 1A and 2A classes, were active on c-Myc/Max proteins. Heparin was more effective in inhibiting DNA binding in control samples than in TCDD-treated samples, i.e. it is likely that CKII was clearly down-regulated in TCDD samples as compared with corresponding control samples.

According to Meek and Street [17], Myc protein is phosphorylated by two major nuclear kinases, CKII and GSK3. The former phosphorylates two of the most conserved regions of Myc, an acidic central region [240–262] and a segment flanking the basic DNA-binding domain [342–357] both *in vitro* and *in vivo*, and it is considered, therefore, that CKII significantly affects DNA binding. The site of GSK3 phosphorylation is within the proline/glutamate-rich region, but its significance is not known. MAP kinase is also known to phosphorylate at Ser-62. This region is known to be needed for transcription of c-Myc activation. Serine is the predominant amino acid phosphorylated by those kinases and, therefore, serine phosphatases must play some regulating role in concentrating the above-mentioned phosphorylation signaling. The overall effect of TCDD on this transcription factor is down-regulation of its DNA binding, to which reduced CKII is likely to be a contributing factor.

Unlike the case of c-Myc, the effect of TCDD on binding to AP-1 response element DNA was up-regulated consistently at both time points studied. In the presence of ATP and Mg^{2+} (Fig. 8, treatment group 3), however, heparin suppressed this effect of TCDD completely. The presence of vanadate and okadaic acid (treatment group 4) made no difference to this action of heparin, indicating that, at this early time point, CKII likely plays a major role in transmitting the effect of TCDD to DNA binding of AP-1. An as-of-yet unexplainable aspect of this observation is that at

this time point, the level of activity of CKII in TCDD-treated samples already has been shown to be lower than control (Fig. 4), rather than higher. An inevitable conclusion, therefore, is that regulation of AP-1 binding activity through phosphorylation is much more complex than it appears. Meek and Street [17] indicated that MAP kinase, GSK3, cyclin-dependent kinase, protein kinase C and protein kinase A are important kinases in regulating c-Jun and c-Fos. Thus, the role of CKII in the regulation of these two AP-1 proteins could be interactive, or an indirect one such as activation or inactivation of any of the above kinases. Moreover, there are other kinases as well as additional members of the AP-1 family of proteins, which are known to be regulated by phosphorylation. The best course of action, therefore, appears to be to treat this subject as a black box, acknowledging its complex nature. With that in mind, the most clear-cut trend we could observe was the disappearance (Fig. 8, treatment groups 3 and 4) of the stimulatory effect of TCDD when heparin was added to these samples during incubation.

In conclusion, we showed clearly that TCDD affected activities of protein kinases and phosphatases in the nuclei of guinea pig hepatocytes. We confirmed our previous finding that down-regulation of heparin-sensitive CKII is a prominent feature of hepatic nuclei from TCDD-affected animals. Additionally, we demonstrated that the phosphorylation status of c-Myc and AP-1 was a strong determinant of their DNA-binding activities. Knowing that the important functions of many nuclear transcription factors are often coordinated and orchestrated by nuclear protein kinases and phosphatases, our current results offer the initial indication of the presence of a fruitful area of research, and furthermore, they provide the impetus to start a search for the causative nuclear phosphorylation events responsible for the myriad of biochemical changes this compound is known to induce.

This work was supported by ES05233 and ES05707 from the National Institute of Environmental Health Sciences, Research Triangle Park, NC. We thank Debra Y. Dunlap for critically reading this manuscript.

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