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Effect of the Histone Deacetylase Inhibitor Trichostatin A on the Responsiveness of Rat Hepatocytes to Dioxin

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ABSTRACT. Since histone acetylation has been implicated in the facilitation of specific gene transcription, we investigated the effect of increasing histone acetylation through inhibition of histone deacetylase on 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) induction of P4501A activity in cultured rat hepatocytes. Inhibition of histone deacetylation was accomplished with addition of trichostatin A (TSA) to the incubation medium, and P4501A activity was measured spectrofluorometrically by determination of the rate of resorufin formation by ethoxyresorufin-O-deethylase (EROD). While TSA alone (5–200 ng/mL) had no effect on EROD activity, TSA potentiated the effect of various concentrations (10^{-12} to 10^{-10} M) of TCDD. Addition of 200 ng TSA/mL with TCDD resulted in an increased EROD activity of ~200% compared with TCDD alone. When TSA was removed from the cells after various incubation times (2, 6, 24 hr) by successive washings with TSA-free medium, it was determined that TSA was required for 24 hr in order to potentiate the effects of a 48-hr incubation with TCDD. In addition to measurement of EROD activity, P4501A1 and 1A2 microsomal protein were determined by western immunoblotting analysis. While neither P4501A1 nor 1A2 was detectable in the presence of TSA alone, P4501A1 was present after incubation of cells with TCDD in the presence or absence of TSA. TCDD plus TSA also resulted in the formation of P4501A2. The results of this study suggest an important role for histone acetylation in the action of TCDD on induction of P4501A enzymes. BIOCHEM PHARMACOL 53;7:951–957, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. histone deacetylase inhibition; TSA; rat hepatocytes; dioxin

TCDD[‖] is an environmental contaminant whose potential toxicity concerns residents in many areas of the world [1]. TCDD activates the expression of the cytochrome P450 family of genes [2]. The cytochrome P450 enzymes are a superfamily of isoenzymes involved in detoxifying environment pollutants such as benzo[*a*]pyrene (found in tobacco smoke and charcoal-broiled meat), polychlorinated biphenyls (used in insulating materials), and dioxin (TCDD), a by-product of the production of commonly used defoliants and combustion in general [3]. The P4501A family includes two genes, *CYP1A1* and *CYP1A2*, and their expressions are induced by TCDD. It is generally assumed that TCDD enters cells by passive diffusion, where it encounters and binds the cytosolic AhR. The receptor–ligand complex is transformed to a DNA-binding form that can enter the nucleus. The complex then dimerizes with ARNT. Interaction of the TCDD–AhR complex with specific DNA sequences (DREs) results in the enhanced transcription of *CYP1A1* and *CYP1A2* genes [4]. This allows the synthesis of the mRNA that is the template for the synthesis of the cytochrome P4501A1 and 1A2 enzymes [3].

Histones, once thought to serve as nothing but cellular packing materials for chromatin, have been implicated as vital participants in both repressing and facilitating activation of many genes [5]. They help to initiate the copying, or transcription, of information stored as DNA into molecules of RNA. In cells, histones are the main components of nucleosomes. DNA wraps roughly twice around the nucleosomal core, which is composed of histones H2A, H2B, H3, and H4. Nucleosomes often form at the TATA box (transcription initiation site). Activation proteins at upstream activator sequences directly or indirectly cause the histone core particles to dissociate from the initiation site. Therefore, the DNA strands are opened and transcription is initiated. It has been observed that transcription is often accompanied by the addition of acetyl (CH_3CO) to histone tails. Such additions would be expected to neutralize the positively charged tails, which in turn could potentially disrupt the interaction of tails with negatively charged

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‖ *Abbreviations:* TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TSA, trichostatin A; EROD, ethoxyresorufin-O-deethylase; AhR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon translocator protein; and DRE, dioxin responsive elements.

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DNA and free TATA boxes from nucleosomes, and initiate transcription. The acetylation and deacetylation of histones are very important for gene transcription, and they are controlled by an equilibrium between the activities of two specific enzymes, histone acetyltransferase and deacetylase [5]. TSA was found to be a very specific inhibitor of histone deacetylase [6]. It was found that histones in cells treated with TSA were acetylated to an unusually high extent. Pulse-chase experiments showed that histone hyperacetylation induced by TSA was not due to increased acetylation but to decreased deacetylation of histones. The very low effective concentration and strict structural specificity of TSA enable its use as a well-defined biochemical probe for histone acetylation. Thus, the inhibition of histone deacetylation and the resulting histone acetylation should result in opening up of the DNA strands and recruitment of transcription factors such as TCDD–AhR complex and other transcription factors so that *CYP1A1* and *CYP1A2* gene expression is activated. Recent studies from our laboratory have demonstrated the *in vitro* interaction of the Ah receptor with a subset of histone H4 [7]. This interaction could increase accessibility of DREs and enhance gene expression.

The experiments described in this manuscript address the effect of histone acetylation on dioxin responsiveness in the intact cell. Primary cultures of adult rat hepatocytes have been used as a unique system for the study of many liver functions including induction of cytochromes P450. The EROD assay is a well-established assay to measure cytochrome P450 activity [8]. In this paper, we have used a primary culture system and the EROD assay to study the effect of TSA on TCDD responsiveness.

MATERIALS AND METHODS

Chemicals

All chemicals were from the Sigma Chemical Co. (St. Louis, MO) unless specified. TCDD was a gift from Dr. S. Safe and was dissolved in DMSO. The purity of TCDD was >99% as determined as previously described [7]. TSA, a gift from Dr. Yoshida, was purified by HPLC with methanol:water (7:3, v/v) as the solvent system. All dilutions were made in complete medium, and the final DMSO concentration was less than 0.01%.

Hepatocyte Isolation and Culture

Mature male Sprague–Dawley rats (250–300 g) (Harlan Sprague–Dawley, Indianapolis, IN) were housed individually in stainless steel cages. Purina Certified Rat Chow and water were provided *ad lib*. Rat hepatocytes were isolated by using a procedure established previously [9]. The procedure used a two-step collagenase (1%, type I, Boehringer Mannheim, Indianapolis, IN) perfusion technique. The viability of hepatocytes was assayed by trypan blue exclusion. After isolation, hepatocytes were placed on six-well 35-mm or 100-mm plastic tissue culture plates (Costar, Cambridge, MA) that were precoated with type I rat tail collagen. Hepatocytes were plated at 1×10^6/well or 5×10^6/plate. The culture medium used for hepatocyte culture was Waymouth 752/L medium (Gibco, Long Island, NY) supplemented with 11.2 mg/L alanine, 12.8 mg/L serine, 24 mg/L asparagine, 2 g/L fatty acid poor serum albumin, 0.168 mg/L aminolevulinic acid, 5 mg/L oleic acid, 5 mg/L *d,l*-tocopherol, 0.393 mg/mL dexamethasone, 7.9 mg/L *d*-thyroxine, 0.03 mg/L glucagon, 20 U/L insulin, and 84 mg/L gentamicin. Cells were kept at 5% CO_2/95% air, 37° and high humidity [8]. After a 2-hr attachment, the plating medium was removed and fresh medium was added.

Assay for EROD Activity

After 24- or 48-hr treatment with TCDD, hepatocytes were washed twice with Krebs-Ringer buffered salt solution and then incubated with 1 mL Krebs-Ringer buffered salt solution containing 10 μM ethoxyresorufin and 10 μM dicumarol for 30 min. EROD activity was measured spectrofluorometrically by determination of the rate of resorufin formation as previously described [7]. Hepatocytes were freshly isolated for each of the experimental protocols, resulting in some variability in EROD activity between experiments.

TCDD and TSA Treatment

Medium was changed 24 hr after the hepatocytes were attached. After another 24 hr, fresh medium containing 200 ng TSA/mL was added, and the hepatocytes were incubated for 30 min prior to the addition of various concentrations of TCDD for 48 hr. Treatment was performed in triplicate wells and EROD activity was assayed as described above. In another set of experiments, different concentrations of TSA were added to the cells 30 min prior to the addition of 10^{-12} M or 10^{-10} M TCDD.

To assess the effect of time of TSA treatment on TCDD responsiveness, hepatocytes were preincubated in 200 ng TSA/mL for 60 or 30 min prior to the addition of 10^{-8} M TCDD, or TSA was added at 0, 30, or 60 min after the addition of 10^{-8} M TCDD. Cells were incubated with 10^{-8} M TCDD for a total of 24 or 48 hr. To further test the time effect of TSA plus TCDD on TCDD responsiveness, hepatocytes were preincubated with 200 ng TSA/mL for 30 min prior to the addition of 10^{-12} M TCDD. TSA remained in medium for 0.5, 2, 6, or 24 hr, at which time the cells were washed with fresh medium to remove TSA and the incubation was continued with TCDD for a total of 48 hr. Results were compared with those of cells incubated with 10^{-12} M TCDD plus or minus TSA for 48 hr.

Western Blot Analysis

To determine the effect of TCDD with or without TSA on P4501A1 and 1A2 production, rat microsomal proteins were isolated from control, 10^{-12} M TCDD treated, 200 ng TSA/mL treated, and 10^{-12} M TCDD plus 200 ng TSA/mL

treated cultured hepatocytes. Proteins were separated on a 12% polyacrylamide gel and electrotransferred onto a PDVF membrane (Immobilon P, Millipore, Marlborough, MA). P4501A1 and 1A2 were detected using anti-rat cytochrome P4501A1 and 1A2 and the ECL Western Blotting Kit obtained from the Amersham Corp. (Arlington Heights, IL).

Statistics Analysis

ANOVA was used with the alpha level set at 0.001.

RESULTS

Effect of TSA on EROD Induction by TCDD

Various concentrations of TCDD (10^{-12} M to 10^{-10} M) led to a significant increase in EROD activity for all concentrations tested ($P < 0.001$) (Fig. 1). The maximum induction activity was 6-fold with 10^{-10} M TCDD. TSA (200 ng/mL) had no effect on EROD activity, but addition of the same concentration of TSA with different concentrations of TCDD (10^{-12} M to 10^{-10} M) resulted in a greater increase in EROD activity for all concentrations of TCDD ($P < 0.001$). At 10^{-12} M TCDD, addition of TSA in this set of experiments increased EROD activity approximately 100% compared with 28% for 10^{-10} M TCDD. Therefore, the effect of TSA was greater at sub-maximal concentrations of TCDD. Increasing concentrations of TSA (5–200 ng/mL) alone did not lead to significant induction of EROD activity (Fig. 2). However, EROD activity in the presence of TSA (100 or 200 ng/mL) plus 10^{-12} M TCDD was increased significantly (Fig. 2, top panel) compared with 10^{-12} M TCDD alone. Addition of various concentrations of TSA with 10^{-10} M TCDD led to a greater total increase in EROD induction activity compared with 10^{-12} M TCDD, and addition of 200 ng TSA/mL caused an ~200% increase in EROD activity compared with TCDD alone (Fig. 2, bottom panel).

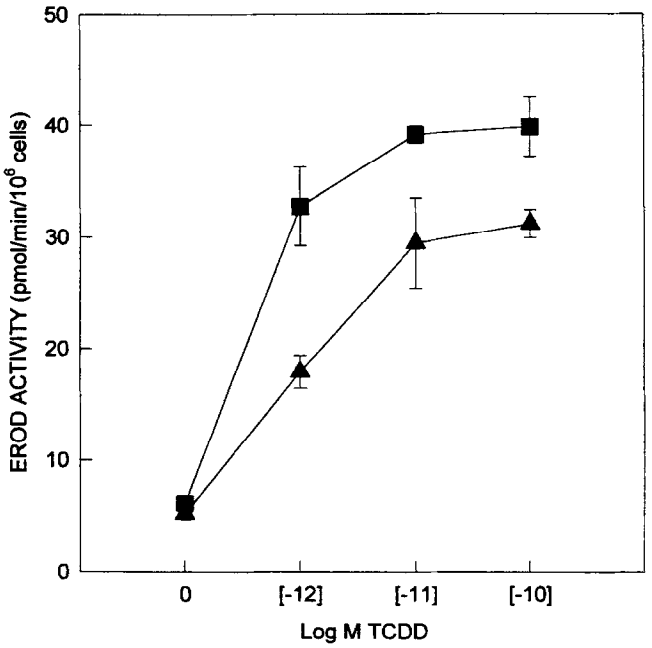

FIG. 1. Effect of TSA on EROD induction by various concentrations of TCDD. Rat hepatocytes were incubated with 10^{-12} M to 10^{-10} M TCDD, in the absence (▲) or presence (■) of 200 ng TSA/mL. TSA was administered 30 min prior to the addition of TCDD. EROD assay at 48 hr was used to measure the induction of P4501A by TCDD. Data are expressed as the means ± SEM of at least three independent measurements.

Effect of Time on TSA Potentiation of TCDD Responsiveness

To determine whether TSA was more effective if added prior to TCDD, experiments were performed with TSA addition 60 min prior or after addition of TCDD with TSA remaining in the incubation medium throughout the 24- or 48-hr incubation with TCDD. TSA potentiated the effect of TCDD which increased EROD induction activity during both the 24-hr (Fig. 3, top panel) or 48-hr (Fig. 3, bottom panel) incubation period. In the presence of TCDD alone, EROD activity was greater at 48 hr than at 24 hr; however, 200 ng TSA/mL potentiated TCDD responsiveness in the 24-hr treatment groups as well as in the 48-hr treatment groups. In these experiments, TSA, regardless of when added, remained in the medium until EROD activity was measured. To determine if a pulse of TSA was sufficient to potentiate TCDD responsiveness, TSA was removed by extensive washings after 0.5, 2, 6 and 24 hr (Fig. 4), with TCDD treatment continuing for 48 hr. Only after 24-hr TSA incubation with TCDD did TSA enhance the TCDD effect. Compared with TCDD alone, the induction activity in the 24-hr TSA treatment group was increased 2-fold compared with 4-fold when TSA was present throughout the 48-hr period.

Effect of TSA on Cytochrome P4501A1 and 1A2 Protein

Hepatocytes were treated with 200 ng TSA/mL, 10^{-12} M TCDD, and TSA plus 10^{-12} M TCDD for 48 hr. Microsomal protein was isolated from cells from each treatment group. Compared with the control, TSA treatment did not induce P4501A1 or 1A2 protein, but TCDD and TCDD plus TSA both increased P4501A1 protein. Interestingly, TSA plus TCDD also caused an increase in P4501A2 protein (Fig. 5).

DISCUSSION

Histones, once thought to serve as nothing more than DNA packing materials, play an important role in both repressing and facilitating activation of many genes [10]. Each of the core histones, H2A, H2B, H3, and H4, have been implicated as regulatory factors either alone or in concert with other histones. Several studies have shown that

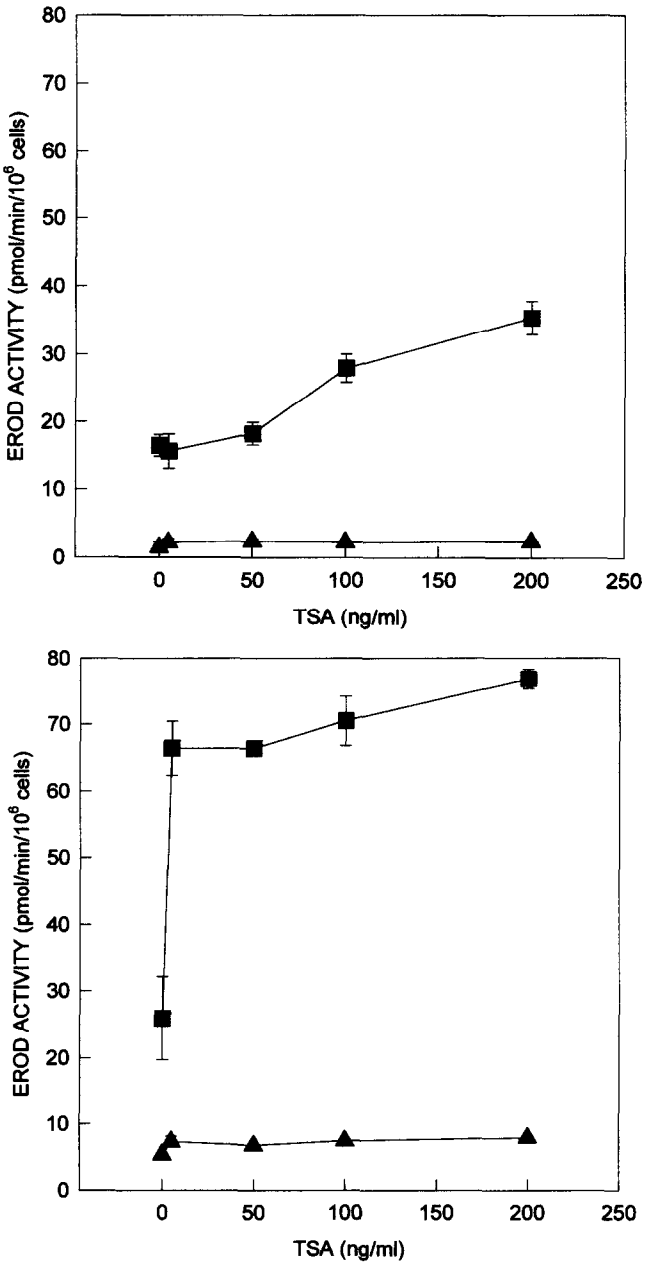

FIG. 2. Effect of various concentrations of TSA on EROD induction by TCDD. Rat hepatocytes were treated with 5–200 ng TSA/mL in the absence (▲) or presence (■) of 10^{-12} M (top panel) or 10^{-10} M (bottom panel) TCDD. TSA was added 30 min prior to the addition of TCDD. EROD assay at 48 hr was used to measure P4501A activity induced by TCDD. Data are expressed as the means ± SEM of at least three independent measurements.

histone acetylation activates gene expression [11]. Increased histone acetylation has been reported to have effects on the expression of specific genes, and does not appear to affect gene expression generally [12–14]. The level of histone acetylation is controlled by an equilibrium between two enzymes: histone acetyltransferase and histone deacetylase. TSA, which is an inhibitor of histone deacetylase, recently has become an important tool for studies of histone acetylation [6, 15]. TSA was first discovered as a microbial metabolite that was effective as an antifungal antibiotic [16]. TSA in low concentrations was shown to activate different genes such as endo A, gelsolin, and H1° [17–19]. TSA was also shown to induce cell differentiation [20] as well as inhibit the cell cycle [21]. Earlier studies utilized high concentrations of sodium *n*-butyrate, a non-competitive inhibitor of histone deacetylase [22]. However, the concentrations of butyrate required to alter histone acetylation had to be interpreted with caution, since such high concentrations affected many cellular enzymes, the

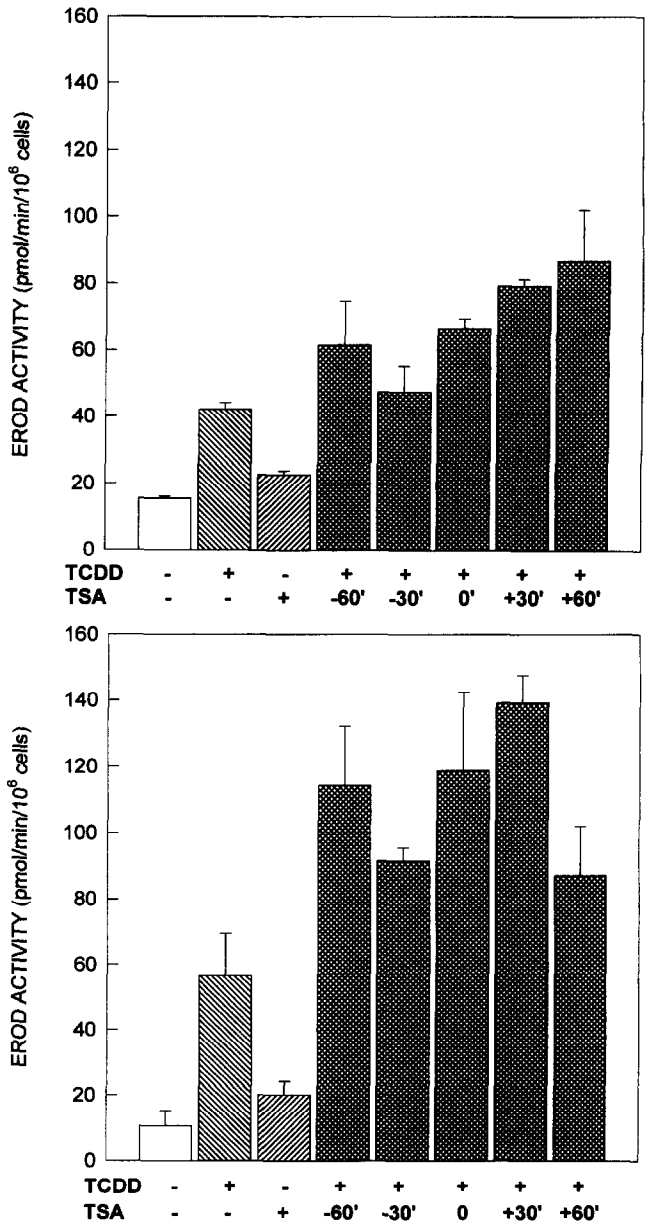

FIG. 3. Effect of time of addition of TSA on EROD induction by TCDD. TSA (200 ng/mL) was added at −60 min to +60 min, with TCDD (10^{-8} M) being added at 0 time. Incubation with TCDD continued for 24 hr (top panel) or 48 hr (bottom panel). Data are expressed as the means ± SEM of at least three independent measurements.

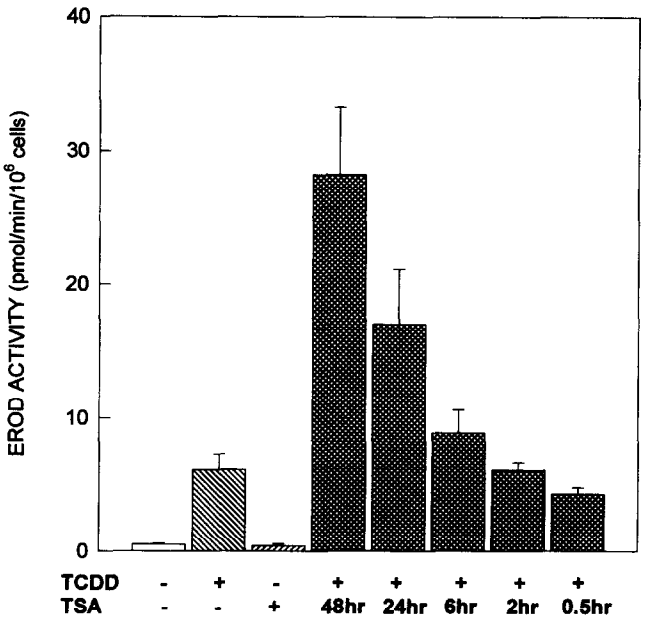

FIG. 4. Effect of length of TSA exposure on EROD induction by TCDD. After rat hepatocytes were treated with 200 ng TSA/mL for 0.5, 2, 6, or 24 hr, TSA was removed by washing with fresh medium. TCDD (10^{-12} M) was present throughout the 48-hr period prior to EROD measurement. Data are expressed as the means ± SEM of at least three independent measurements.

cytoskeleton, and cell membranes. Therefore, recent studies with TSA, a specific inhibitor of histone deacetylase, have yielded important new information on the effects of histone acetylation on cell proliferation and differentiation.

In our study, we examined the effects of TSA on TCDD responsiveness of rat hepatocytes in culture as measured by the EROD assay, an indicator of P4501A activity. In most experiments the effect of TSA was greater at submaximal concentrations of TCDD. The higher TSA concentrations (100–200 ng/mL) caused a greater EROD induction by TCDD. These results suggest that TCDD responsiveness can be potentiated by the inhibition of histone deacetylation, i.e. hyperacetylating histones, which results in activation of CYP1A expression. We also examined the effect of time on TSA potentiation of TCDD responsiveness. TSA potentiation of TCDD induction of CYP1A1 appears to require the continued presence of TSA. Presumably, the increased acetylation of histones can relax nucleosomes and, thereby, the chromatin structure, keep the DNA strands open, and enhance the binding of transcription factors to specific DNA sequences. Addition of TSA to the hepatocytes within an hour prior to or subsequent to the addition of TCDD resulted in the same potentiation of TCDD effects as long as the TSA remained in the culture medium. Our studies suggest that TSA is required for at least 24 hr in order to enhance TCDD induction of EROD activity.

High levels of histone acetylation are associated with transcriptionally active chromatin [23, 24]. In general, as the N-terminal lysines of histones become acetylated, the positive charge property of this region is lost, and the affinity that this portion of the molecule has for negatively charged DNA is reduced [25]. Although a multitude of studies have been performed on each of the individual histones, H4 is one of the best studied. The important functional role of the N-terminus of H4 may lie in the control of transcription, specifically in modulating the access of transcription factors [26]. We have found that subsets of this histone interact with ligand-activated intracellular receptors. The rabbit liver AhR was found to bind with high affinity to a subset of histone H4 that was tightly bound to DNA and resistant to salt extraction [7]. We also recently reported the interaction of the uterine estrogen receptor to variants or modified histones H2B and H4 [27]. Although in the present studies we did not examine the acetylation of individual histone components, it is likely that TSA promoted the acetylation of several histones, including H4. Such modifications could then enhance the interaction of the TCDD-AhR complex with the respective DREs, resulting in a potentiation of *CYP1A1* and *CYP1A2* gene expression. Alternatively, increased histone acetylation could increase the expression of a gene encoding a transcription factor that, in turn, would allow the potentiation of dioxin effects on *CYP1A* gene expression. It has been reported that butyrate induces c-*fos* gene expression [12, 28] while inhibiting the transcriptional activation function of MyoD [14].

It has been postulated that binding of AhR–ARNT to DREs within the enhancer region of the *CYP1A1* gene mediates an alteration of local chromatin structure [29]. It has been demonstrated that treatment of cells with TCDD caused an increase in accessibility of the *CYP1A1* promoter [30]. This enhanced accessibility was rapid in onset and independent of transcription of the *CYP1A1* gene. The enhancer region covers about 700 base pairs and includes 6

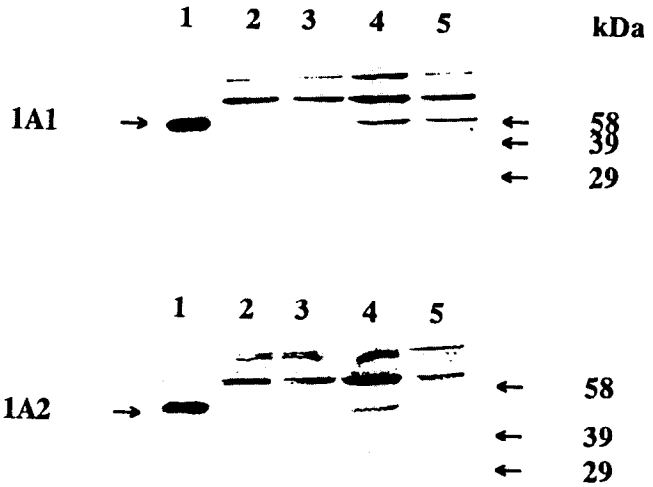

FIG. 5. Measurement of P4501A1 and 1A2 by western analysis. Standard P4501A1 (lane 1-top) or P4501A2 (lane 1-bottom) were run on SDS–PAGE alone with 5 µg microsomal protein from rat hepatocytes treated with vehicle (lane 2), 200 ng TSA/mL (lane 3), 10^{-12} M TCDD plus TSA (lane 4), or 10^{-12} M TCDD (lane 5).

DREs. Prior to treatment of cells with TCDD, the major groove was largely devoid of interaction proteins. The inactive enhancer is apparently free of *trans*-acting factors as measured in methylation protection assays; however, upon treatment with TCDD, the enhancer region bound the AhR:ARNT complex at all 6 DREs. Additionally, it has been observed that the enhancer region of the uninduced *CYP1A1* gene is in a nucleosomal configuration which necessitates that the AhR:ARNT interact with nucleosomes [31]. Thus, binding of the AhR:ARNT complex to DREs within the enhancer may mediate an alteration of local chromatin structure, thereby allowing increased promoter accessibility and transcriptional activation.

The interaction between the AhR complex and histone H4 suggests that AhR may facilitate the disruption of nucleosomal structure by altering the conformation of H4, which could lead to destabilization of the nucleosomal core structure. It has been proposed that the interaction of the AhR complex with nucleosomal proteins could lead to the functioning of or the recruitment of histone acetylase [29], which could modify the properties of the H4 and subsequently mediate increases in enhancer accessibility. It is also possible that interaction of the AhR complex with H4 might stabilize the binding of the receptor complex to DREs, leading to activation of TCDD-inducible gene expression.

Our present study also demonstrated that TSA alone had no effect on EROD activity. In addition, our studies showed that TCDD plus TSA not only increased P4501A1 protein, but also induced P4501A2 protein. In the rat hepatic cytochrome P4501A family, the turnover number for reconstituted rat hepatic cytochrome P4501A1 is 10–20 times larger than that of P4501A2 [32]. P4501A1 accounts for approximately 98% of induced CYP1A activity. Our results suggest that activating gene expression by inhibiting histone deacetylase not only activates *CYP1A1* gene expression but also induces the *CYP1A2* gene. This observation will be explored in future studies.

Thus, our studies suggest an important role for histone acetylation in the action of TCDD on induction of P4501A enzymes. The series of events involved in ligand–receptor activation of genes appears to require alterations in chromatin structure, which may allow access of the receptor complex to elements within DNA.

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