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TCDD (2,3,7,8-TETRACHLORODIBENZO-P-DIOXIN) CAUSES INCREASES IN PROTEIN KINASES PARTICULARLY PROTEIN KINASE C IN THE HEPATIC PLASMA MEMBRANE OF THE RAT AND THE GUINEA PIG

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SUMMARY: To study the cause of TCDD-evoked changes in the functions of plasma membrane constituents TCDD's effects on protein kinase activities in the liver of rats and guinea pigs were investigated. TCDD was found to cause a sharp increase in both c-AMP independent and dependent protein kinase activities in plasma membrane preparations from rat liver within 48 hours from the time of administration. Such effects reached maxima around day 20, and were quite noticeable even 40 days after a single administration of TCDD. As a result of SDS-polyacrylamide gel-electrophoresis (SDS-PAGE) analysis several substrate proteins for these increased protein kinases were observed. Among them are 170 K - 150 K bands, representing EGF receptor protein. TCDD was found to particularly stimulate protein kinase C which is known to influence many enzyme and receptor functions through protein phosphorylation. The possible significance of such an action of TCDD is discussed.

TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) is a very toxic environmental pollutant. It is also an acnegenic, teratogenic and carcinogenic chemical (1). However, the molecular basis of its toxicity is not known. Recently it has been reported from this laboratory that in vivo administration of TCDD affects the ligand binding activities of EGF (2) (epidermal growth factor) and LDL (low density lipoproteins) (3) receptors in the hepatic plasma membranes of rats and guinea pigs. Furthermore, TCDD was shown to cause reduction in the levels of insulin receptor (2), concanavalin A binding (4) and ATPases (2,5,6) of the hepatic plasma membrane of rats.

The cause for such simultaneous changes in functions of the plasma membrane constituents is unknown. Previously we have observed that the total protein kinase activities in the plasma membrane preparation from the TCDD-treated rats were much higher than that from the untreated ones. Accordingly, one of the working hypotheses we have considered has been the involvement of some protein kinases evoking pleiotropic changes in affected cells. In this paper we report our finding that TCDD causes increases in various protein kinases, particularly protein kinase C in the hepatocytes from rats and guinea pigs.

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METHODS

TCDD was dissolved in corn oil:acetone (9:1) and intraperitoneally injected to male Sprague-Dawley rats (150 - 200 g) or guinea pigs (English shorthair type 200 -250 g) at single dosing of 25 μg/kg and 1 μg/kg, respectively (2). After given time periods the animals were sacrificed, and hepatic plasma membranes were obtained as before (4). The assay method used to prepare phosphorylated endogenous membrane proteins for electrophoresis was that of Rubin et al. (7) with approximately 10 μCi of gamma-^{32}P-ATP (10 μM, 1 min. incubation), and SDS-PAGE (7.5% polyacrylamide gel-electrophoresis) was developed following Laemmli method (8). Protein kinase C in the hepatic plasma membranes was studied with histone according to the method of Kishimoto et al. (9), Takai et al. (10) and Niedel et al. (11) with following modifications: the hepatic plasma membrane prepared as above (2,4) was directly homogenized in the EGTA-EDTA solution (11), centrifuged to remove undissolved membrane, and the supernatant was used as an enzyme source. The activity of general c-AMP dependent and independent protein kinases was determined by the method of Corbin and Reiman (12). Histone was used as an artificial substrate.

RESULTS AND DISCUSSION

The time-response relationships of changes resulting from TCDD administration in vivo in general protein kinase levels in the rat hepatic plasma membrane are shown in Figure 1. The maximum stimulation occurred on day 20 of post treatment for both classes of kinases, but the overall degree of stimulation was higher for c-AMP independent kinases than for the dependent ones. The degree of TCDD stimulation was more pronounced (12-fold on day 20) for the former than for the latter (4.5-fold on day 20). Under our experimental conditions there was some mortality (20-30%) between day 21 and day 40. Therefore, the apparent recovery observed on day 40 (Figure 1A) may not reflect a true recovery as the data were generated by studying only surviving subpopulations.

To study the nature of native protein substrates for increased protein kinases hepatic plasma membrane preparations from TCDD treated and control rats and guinea pigs were incubated (7) with gamma-^{32}P-ATP without histone and resulting phosphoproteins were analyzed on SDS-PAGE. It was noted immediately that the intensities of a number of bands in preparations from TCDD-treated animals were high in the resulting autoradiogram (Fig. 2). The bands of which intensity increased were: for guinea pigs 270 K, 170 K, 118 K, 90-95 K, 80-85 K, 68 K, 58-64 K, 53 K, 41 K and 35 K and for rats 270 K, 170 K, 150 K, 130 K, 115 K, 98 K, 87 K, 77 K, 67 K, 55-58 K, 53 K, 48-50 K, 37-40 K and 33 K daltons. On the other hand, the band intensity was decreased for 54 K for guinea pigs and 84 K region for rats.

The intensified band region at 170 K corresponds to EGF receptor which is often accompanied with 150 K band representing its degradation product (13). We have shown previously that TCDD, when administered in vivo, causes reduction in number of EGF receptors in the hepatic plasma membrane in guinea pigs and rats (2,14). One of the agents which is known to cause reduction in the number of EGF receptors is TPA (12-0-tetradecanoyl phorbol-13-acetate). This cancer promoter acts in this manner by activating TPA receptor associated protein kinase C (11). Since there are a number of

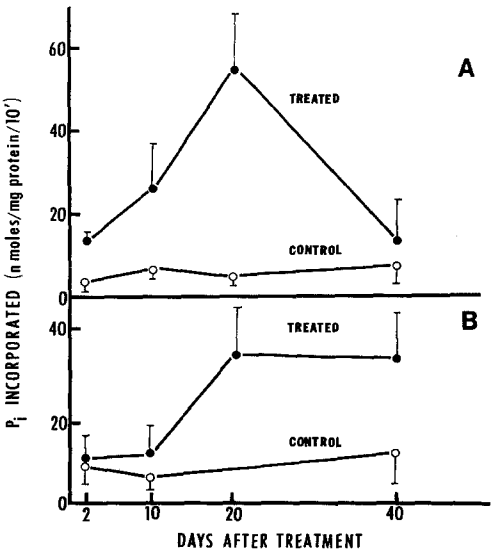

Fig. 1. Time course of changes in protein kinase activities in the hepatic plasma membranes from male Sprague-Dawley rats treated with 25 µg/kg (single, intraperitoneal injection) of TCDD. (A) c-AMP independent protein kinases, and (B) c-AMP dependent protein kinases. Control rats received the same volume of vehicle (corn oil and acetone, 9:1) only. At given time periods, the animals were sacrificed and their livers were processed for plasma membrane.[4] Protein kinase activity was measured according to Corbin and Reimann[12] using 50 µg plasma membrane protein in 50 µl 0.25 M sucrose. After incubating for 10 minutes at 30°C the reaction was stopped with 3 ml cold trichloracetic acid (10%) followed by 100 µl water, containing 1 mg bovine serum albumin (BSA) and 1.36 mg KH_2PO_4. The tubes were allowed to stand for 5 minutes for complete protein precipitation, spun for 5 minutes (1000 x g), the supernatant decanted, and the pellet redissolved with 0.5 ml NaOH (0.2N). Reprecipitation with trichloroacetic acid and a second centrifugation followed. After repeating the washing procedure once more (total of 2 times) the final pellet was resuspended with 0.3 ml formic acid, of which 0.2 ml was used for liquid scintillation counting.

similarities between the biological effects caused by TCDD and TPA, an attempt was made to study whether some of the stimulatory effects of TCDD on phosphorylation activities are due to its activation of protein kinase C.

Protein kinase C activity was defined as the portion of kinase activity which is stimulated by calcium and phosphatidylserine (9,10,11). Since the properties of protein kinase C in the liver tissues have not been well documented, we have first examined the effects of agents, which are known to stimulate protein kinase C in other tissues on the EGTA-EDTA solubilized enzyme preparation from rat hepatic plasma membrane. It is clear from the results (Fig. 3) that protein kinase C in this hepatic preparation responds to these agents in a manner consistent with the description of this enzyme from other tissues (10,11). As expected, TPA's stimulatory effect was particularly significant.

We have also examined the levels of protein kinase C in various preparations from TCDD-treated and control animals. The results (Table 1) clearly indicate that

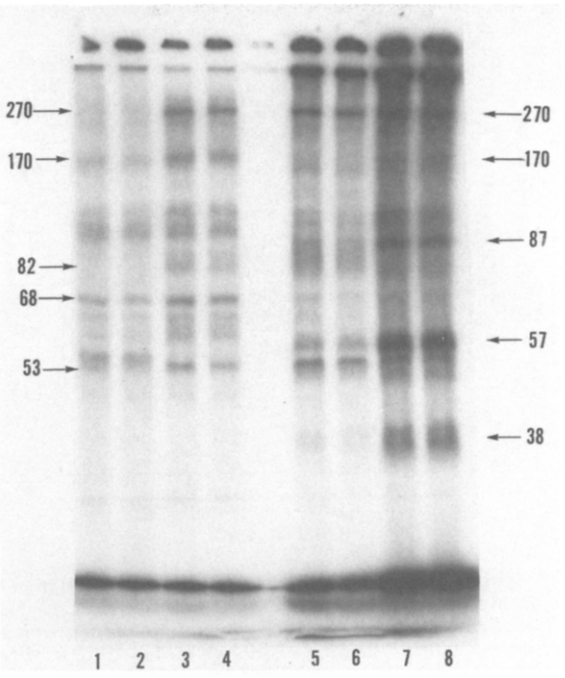

Fig. 2. SDS-electrophoretogram/radioautogram of hepatic plasma membrane proteins phosphorylated with gamma ^{32}P-ATP from control (Lane 1,2) and TCDD-treated (25 µg/kg single i.p.) guinea pigs (Lane 3,4) and control (Lane 5,6) and treated (25 µg/kg single i.p.) rats (Lane 7,8). To obtain the radioautogram, the hepatic plasma membrane preparations were isolated from the control and treated animals after 10 days from the time of TCDD injection, and incubated directly with gamma-^{32}P-ATP without histone as described by Rubin et al.[7] The reaction was stopped with addition of SDS, the level of total protein for each electrophoresis test was adjusted and electrophoresis was developed.[8] Thereafter, the gel was dried and exposed to Fuji X-ray film for radioautography for 7 to 14 days. The positions of phosphorylated EGF receptor (170 and 150 kD) and insulin receptor (subunit, approx. 90 kD) were determined by incubating fresh rat hepatic plasma membranes with non-labeled EGF and insulin, respectively, in a separate experiment (data are not shown).

the levels of protein kinase C are higher in the membrane fractions from TCDD-treated rats and guinea pigs.

In the current investigation we have demonstrated that TCDD administered in vivo causes a profound increase in protein kinase activities in the hepatic plasma membrane of rats and guinea pigs. Two of the kinases activated in the process have been identified to be protein kinase C and EGF receptor.

The pleiotropic nature of protein kinase-induced cellular responses has been pointed out by many scientists. This includes those activated by Rous sarcoma virus (15,16) and other transforming viruses (17), phorbol esters (18,19), growth factors including EGF, platelet derived growth factors and transforming growth factors (20) and some hormones (21). Particularly affected are cell surface receptors. Changes in the cell surface characteristics that are induced by chemical cancer promoters and transforming virus are well known.

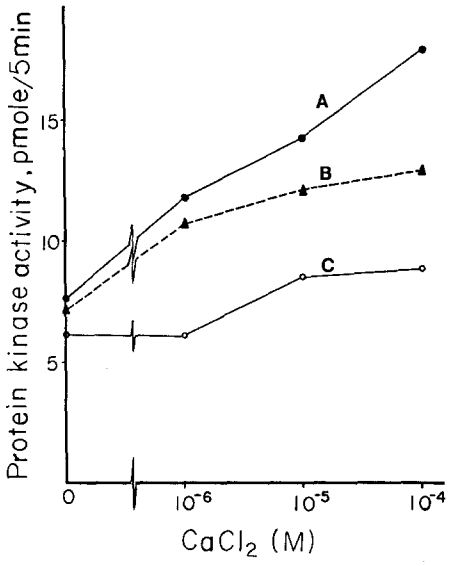

Fig. 3. Stimulatory effects of Ca^{2+} on the protein kinase C activity in the rat hepatic plasma membrane in the presence of (A) TPA + phosphatidylserine (PS), (B) diolein + PS, and (C) PS alone. Protein kinase C in the plasma membrane was solubilized with EGTA-EDTA and assayed as described by Niedel et al. (see Fig. 4 of ref. 11). Kinase activity was expressed as pmole Pi incorporated into histone per 5 min per tube, each tube containing 40 µg membrane protein. The buffer contained 20 mM tris-HCl (pH 7.5), 50 mM β-mercaptoethanol and 2 mM of phenylmethylsulfonylfluoride. The amounts of agents used were: 20 µg/ml PS, 1 µg/ml diolein and 10 µg/ml of TPA. The assay was performed in the presence of 0.7 mM EGTA or added $CaCl_2$ at 10 µM to 0.1 mM incubated at 37°C.

Table I Levels of protein kinase C in the hepatic plasma membrane preparations from control and TCDD-treated rats (25 µg/kg single i.p. assayed on day 10) and guinea pigs (1 µg/kg single i.p. assayed on day 10)

Assay conditions	Protein kinase activity (pmole Pi/5 min/40 µg protein) control	TCDD-treated
Rats (6 animals)		
Basal kinase activity (a)	10.49 ± 2.29	15.65 ± 9.09
Kinase activity in the presence of Ca^{2+} and phosphatidyl-serine (b)	13.64 ± 2.17	23.30 ± 8.90*
Protein kinase C activity (i.e., b-a)	3.15 ± 0.99	7.62 ± 3.60*
Guinea pigs (6 animals)		
Basal kinase activity (c)	3.02 ± 0.72	3.87 ± 0.56*
Kinase activity in the presence of Ca^{2+} and phosphatidyl-serine (d)	4.25 ± 0.47	6.19 ± 0.76*
Protein kinase C activity (i.e., d-c)	1.23 ± 0.69	2.32 ± 1.18

*Significant different against corresponding control values at $P < .05$. All data are presented as mean ± standard deviation determined by using six animals for each test.

Protein kinase C is believed to be a part of the cascade of reactions included in the phosphatidylinositol response (9). While the nature of the specific endogenous protein substrates is largely unknown, it is generally acknowledged that diverse cellular responses to its activator such as TPA may be derived from differences in distribution and functional roles of substrate proteins dictated by differential specialization of various cells. One of the major substrate proteins so far identified in many tissues is the EGF receptor (22). This explains why many of cellular effects of TPA resemble those caused by EGF (18,19). Recently we have also pointed out similarities of TCDD caused cellular changes to those induced by EGF (2,14).

Otherwise, it is not possible, at this stage, to postulate just which of the TCDD-evoked lesions are caused by the elevated levels of protein kinase C. It is probable that TCDD in vivo could stimulate a number of different protein kinases. Therefore, one must be careful in attributing certain lesions solely to one protein kinase.

Whatever may be the cause for TCDD evoked stimulation of protein kinases, it seems worthwhile to pursue this line of investigation in view of the increased awareness of the importance of various protein kinases, including those associated with the EGF receptor in regulating cellular functional and differentiational expressions.

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