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POSSIBLE INVOLVEMENT OF CYCLIC AMP-DEPENDENT PROTEIN KINASE IN *p,p'*-DDT-INDUCED CHANGES IN HEPATIC METABOLISM*

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Abstract—Administration of an acute dose of *p,p'*-1,1,1-trichloro-2,2-bis (*p*-chlorophenyl) ethane (*p,p'*-DDT) (600 mg/kg, p.o.) resulted in a significant increase in hepatic adenylate cyclase activity (40 per cent) and endogenous cyclic AMP levels (31 per cent). Although the cytosol cyclic AMP-independent protein kinase activity was only slightly affected (18 per cent), the activity of the nucleotide-dependent enzyme was significantly decreased to 74 per cent of the control values. Whereas the protein kinase activity ratio (−cyclic AMP/+cyclic AMP) of this soluble enzyme was increased, the ability of the enzyme to bind [^{3}H]cyclic AMP *in vitro* was significantly decreased after pesticide administration. As observed with the soluble enzyme, maximal stimulation of the crude nuclear protein kinase (1500 *g* pellet) was observed 1 hr after *p,p'*-DDT treatment, as indicated by changes in the phosphorylation of endogenous nuclear substrates and cyclic AMP-binding capacity. Studies with lower dosages of DDT revealed that a dose as low as 100 mg/kg was sufficient to produce statistically significant alterations in the activities of both the soluble and particulate enzymes. In most cases, doubling the dosage (200 mg/kg) resulted in greater changes in these biochemical parameters. The present study supports the concept that the DDT-induced alterations in hepatic carbohydrate metabolism may be related to an initial stimulation of the hepatic cyclic AMP–adenylate cyclase–protein kinase system.

In mammals, a variety of cellular functions have been postulated to be initiated through modulation of the adenylate cyclase–cyclic AMP system. The finding that hormones such as glucagon or epinephrine produced a rise in the endogenous levels of hepatic cyclic AMP and that this change preceded hormonal-induced glycogenolysis gluconeogenesis and lipolysis suggested that cyclic AMP is essential to triggering the observed physiological responses [1–4]. Additional support for the role of cyclic AMP as an intracellular messenger in the action of hormones on hepatic carbohydrate metabolism was gained from the observation that the cyclic nucleotide, like glucagon, was capable of producing an increase in blood glucose levels and a fall in liver glycogen content [3, 5, 6]. Cyclic AMP is believed to act within the cell through activation of protein kinase(s). This group of enzymes is now known to control phosphorylase activation and glycogen synthetase inhibition in skeletal muscle [7–9] as well as lipase activation in adipose tissue [10]. In addition, the phosphorylation of ribosomes and histones has been shown to be catalyzed by the cyclic AMP-dependent protein kinase in a variety of animal tissues [11–13]. The aim of the present study was to investigate the influence of *p,p'*-DDT‡ treatment on the activities of cyclic AMP-dependent and -independent protein kinases, protein kinase activity ratio and cyclic AMP-binding capacity of the enzyme in both soluble and crude nuclear preparations.

MATERIALS AND METHODS

Animal and tissue preparation. Mature male rats of the Sprague–Dawley strain weighing approximately 200 g were used in this study. All animals were maintained on Master Laboratory Chow and water *ad lib.* and starved overnight (16 hr) before sacrifice. Rats were given orally various doses of *p,p'*-DDT, and control animals received an equal volume of the vehicle (corn oil). The animals were then sacrificed by cervical dislocation at various time intervals as indicated in the text. The liver was rapidly excised and frozen in liquid nitrogen until the time of the various enzymatic assays.

Biochemical assays. Hepatic cyclic AMP levels, adenylate cyclase and phosphodiesterase activities were determined by methods as described previously [14]. Soluble protein kinase activity (100,000 *g* supernatant) was assayed by measuring the incorporation of ^{32}P into a histone mixture (type IIA, Sigma) as described recently by Tsang and Singhal [15]. The assay medium (final volume 0.13 ml, pH 6.5) contained 46 mM sodium acetate, 1.5 mM NaF, 3.1 mM theophylline, 0.24 mg of histone mixture, 17 mM $MgCl_2$, 85 μM ATP and 5 μM cyclic AMP (for measuring the activity of cyclic AMP-dependent enzyme). The reaction was initiated by the addition of [γ^{32}P] ATP-$MgCl_2$ (2×10^5 cpm) followed by incubation at 30° for 7 min and terminated by the addition of 5 ml of 20% trichloroacetic acid. The protein precipitate was separated by filtering on Millipore filter (HAWP,

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‡ Abbreviations: *p,p'*-DDT or DDT, *p,p'*-1,1,1-trichloro-2,2-bis (*p*-chlorophenyl) ethane; cAMP or cyclic AMP, cyclic 3',5'-adenosine monophosphate.

0.45 μm) and washed three times with the same volume of 5% trichloroacetic acid. The filter was then counted using a scintillation mixture containing 7 parts Omnifluor solution (4 g Omnifluor/liter of scintanalyzed toluene) to 3 parts ethylene glycol monoethyl ether. Kinase activity of the crude nuclear preparation (1500 *g* pellet, washed three times) was assayed by measuring the incorporation of ^{32}P from [$\gamma^{32}P$]ATP into endogenous substrates under the conditions described for the soluble enzyme. Cyclic AMP-binding studies were performed using 0.05 M sodium acetate and 1 mM EDTA, pH 5.0, as described by Sanborn *et al.* [16]. Protein was determined by the method of Lowry *et al.* [17] with bovine serum albumin as standard.

Chemicals. All reagents were of the purest grade available. [^3H]cyclic AMP (28 Ci/m-mole) was purchased from Schwarz–Mann (Orangeburg, N.Y.) whereas [$\gamma^{32}P$]ATP (25.5 Ci/m-mole) and Omnifluor were obtained from New England Nuclear (Dorval, Quebec). The histone mixture (type IIA), ATP and other biochemical intermediates used in various biochemical assays were products of the Sigma Chemical Co. (St. Louis, Mo.).

Statistical analysis. Results are expressed as means ± S. E. M. of five to six animals/group. Differences between the means of the two groups were tested for statistical significance by using the Student's *t*-distribution. No statistical significance is indicated when the P value was >0.05.

RESULTS

Effect of p,p'-*DDT on hepatic cyclic AMP metabolism.* Results presented in Table 1 illustrate the changes in liver cyclic nucleotide metabolism 1 hr after the oral administration of a 600 mg/kg dose of *p,p'*-DDT. Whereas pesticide treatment failed to alter the activity of hepatic phosphodiesterase, a significant increase was noted in the activity of adenylate cyclase (40 per cent) and endogenous cyclic AMP levels (31 per cent). Soluble hepatic protein kinase activity also was stimulated by the pesticide as indicated by an enhancement in the kinase activity ratio (82 per cent) and a decrease in the activity of the cyclic AMP-dependent form of the enzyme (26 per cent). Although protein kinase activity assayed in the absence of cyclic AMP was only slightly increased (18 per cent), the activity of the enzyme in the presence of the nucleotide was significantly decreased from 308.0 to 200.4 pmoles ^{32}P incorporated into the histone mixture/mg of protein.

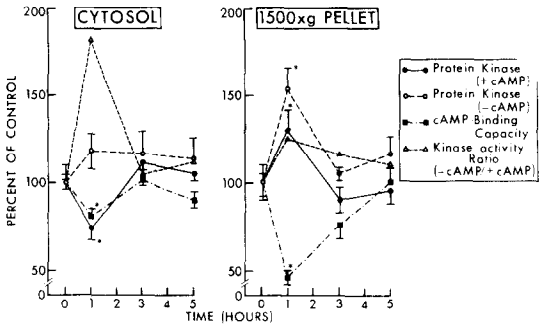

Fig. 1. Time course of the *p,p'*-DDT-induced changes in hepatic protein kinases in cytosol and crude nuclear preparations. Each point represents the mean ± S. E. M. of five to six rats in each group. Animals were given *p,p'*-DDT (600 mg/kg, p.o.) and killed 0, 1, 3 and 5 hr after insecticide treatment. Data are given in percentages taking the values of control rats as 100 per cent. The asterisks indicate statistically significant differences when compared with the values of control rats ($P < 0.05$).

Time course of p,p'-*DDT effect on hepatic protein kinases.* The sequential changes in hepatic protein kinases obtained from both the cytosol and crude nuclear fractions of DDT-treated rats are shown in Fig. 1. One hr after the administration of *p,p'*-DDT (600 mg/kg), both the soluble as well as the particulate protein kinases were maximally stimulated, as indicated by marked changes in the activities of protein kinases, the kinase activity ratio and the cyclic AMP-binding capacity of the enzyme. Whereas stimulation of the soluble enzyme was associated with only a slight enhancement in the activity of the cyclic AMP-independent protein kinase, enzyme activity in the presence of the nucleotide was decreased significantly at 1 hr. These DDT-induced biochemical alterations were paralleled by increments in the dissociation of the soluble holoenzyme as reflected in the increase in kinase activity ratio (from 0.115 to 0.209). As expected, the [^3H]cyclic AMP-binding capacity *in vitro* of the soluble protein kinase, an index of the

Table 1. Effect of *p,p'*-DDT on hepatic cyclic AMP metabolism*

Groups	Adenylate cyclase†	Cyclic AMP†	Phospho-diesterase†	Soluble protein kinase		Kinase activity ratio $\left(\frac{-\text{cyclic AMP}}{+\text{cyclic AMP}}\right)$
				− Cyclic AMP	+ Cyclic AMP	
Control	0.56 ± 0.01 (100)	1.22 ± 0.10 (100)	5600 ± 300 (100)	35.49 ± 4.11 (100)	307.97 ± 3.06 (100)	0.115 ± 0.010 (100)
p,p'-DDT	0.78 ± 0.01 (140)‡	1.60 ± 0.10 (131)‡	5100 ± 200 (91)	41.93 ± 4.21 (118)	200.40 ± 22.79 (74)‡	0.209 ± 0.012 (182)‡

* Each value represents the mean ± S. E. M. of five to six animals in each group. Rats were given *p,p'*-DDT (600 mg/kg, p.o.) and sacrificed 1 hr later. Data are also given in percentage (in parentheses) taking the values of control animals as 100 per cent. Whereas activities of adenylate cyclase and phosphodiesterase were calculated as pmoles cyclic AMP formed or metabolized/mg of protein, protein kinase activity was expressed in pmoles ^{32}P incorporated into histones (type IIA, Sigma)/mg of protein. Kinase activity ratio (−cyclic AMP/+cyclic AMP) was calculated by dividing protein kinase activity assayed in the absence of cyclic AMP by that in the presence of the nucleotide. The concentration of cyclic AMP is given in terms of pmoles/mg of tissue.

† See Ref. 14.

‡ Statistically significant difference when compared with the values of control rats ($P < 0.05$).

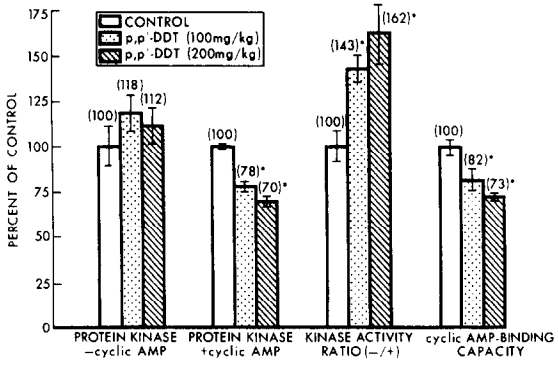

Fig. 2. Influence of two lower dosages of *p,p'*-DDT on soluble hepatic protein kinase and cyclic AMP-binding activities. Each bar represents the mean ± S. E. M. of five to six rats in each group. Animals were treated orally with either a 100 or 200 mg/kg dose of *p,p'*-DDT and sacrificed 1 hr later. Data are given in percentages taking the values of control rats as 100 per cent. The asterisks indicate statistically significant differences when compared with the values of control rats ($P < 0.05$).

saturation *in vivo* of the nucleotide receptor, decreased in an inverse manner, attaining the lowest levels (81 per cent) also at hr 1 after pesticide exposure.

While exogenous cyclic AMP failed to stimulate the particulate enzyme in both control and experimental animals, incorporation of ^{32}P into endogenous nuclear substrates from [$\gamma^{32}P$]ATP was significantly enhanced both in the presence (from 394.3 ± 21.9 to 513.6 ± 49.3 pmoles/mg of protein) and absence (from 358.3 ± 31.1 to 586.1 ± 42.8 pmoles/mg of protein) of the cyclic nucleotide 1 hr after the pesticide administration (Fig. 1). Contrary to the observed independence of the nuclear enzyme for cyclic AMP for its catalytic activity, the ability of the nuclear fraction to bind [^{3}H]cyclic AMP *in vitro* was as high as 11.4 ± 1.1 pmoles of the nucleotide bound/mg of protein (46 per cent of that observed with the soluble enzyme). Moreover, treatment with *p,p'*-DDT resulted in a marked decrease (54 per cent) in cyclic AMP-binding capacity 1 hr after the administration of this halogenated hydrocarbon, although a return to control values was observed by hr 5.

Influence of lower dosages of p,p'*-DDT on soluble hepatic protein kinase and cyclic AMP-binding activities.* Results in Fig. 2 show that a 100 mg/kg dose of *p,p'*-DDT significantly decreased (18 per cent) the cyclic AMP-binding capacity of soluble hepatic protein kinase from 25.0 ± 1 to 20.4 ± 1.6 pmoles [^{3}H]cyclic AMP bound/mg of protein at 1 hr. Treatment with a higher dose of the chlorinated hydrocarbon (200 mg/kg) resulted in an even larger reduction (27 per cent) in the ability of the enzyme to bind the cyclic nucleotide *in vitro*. Whereas administration of *p,p'*-DDT failed to alter the activity of the cyclic AMP-independent protein kinase, a significant reduction (22 per cent) in the activity of the nucleotide-dependent enzyme was observed with the lowest dose used in this study. Increasing the dose to 200 mg/kg resulted in a quantitatively greater decrease (30 per cent) in the activity of this soluble enzyme (Fig. 2). In contrast, the ratio of protein kinase activity in the absence to that in the presence of cyclic AMP was significantly increased (43 per cent) from 0.115 ± 0.010 to 0.165 ± 0.008 in rats given 100 mg/kg of DDT. Treatment with a larger dose (200 mg/kg) produced a greater increase (62 per cent) in the activity ratio of the enzyme.

Effects of p,p'*-DDT on protein kinases in crude nuclear preparation.* In contrast to the hepatic protein kinases from the cytosol, the activity of the particulate enzyme was markedly stimulated in the presence and absence of cyclic AMP after *p,p'*-DDT administration (Fig. 3). While treatment with 100 mg/kg of DDT significantly increased (94 per cent) cyclic AMP-independent protein kinase activity from 358.3 ± 31.1 to 695 ± 85.4 pmoles ^{32}P incorporated into endogenous substrates/mg of protein, somewhat smaller quantitative changes in enzyme activity were observed with the higher dose. Similar findings were noted for the enzyme activity assayed in the presence of the cyclic nucleotide, although the percentage increase after *p,p'*-DDT treatment appeared to be smaller as a result of a slight but insignificant higher value in basal activity (394.3 ± 21.9). Whereas administration of *p,p'*-DDT (100 or 200 mg/kg) failed to significantly alter the kinase activity ratio in the crude nuclear preparation, the cyclic AMP-binding capacity of the particulate enzyme was decreased to approximately 70 per cent of the control values (Fig. 3).

DISCUSSION

Evidence indicates that modulation in the adenylate cyclase–cyclic AMP system may be an essential prerequisite for the organochlorine pesticide-stimulated alterations in liver and kidney carbohydrate metabolism [14, 18]. Kacew and Singhal [14] demonstrated that treatment with α-chlordane, heptachlor or endrin produced stimulation in the activity of adenylate cyclase, elevation in the endogenous levels of cAMP as well as enhancement in the capacity of renal and hepatic tissue slices to biotransform [^{3}H]adenosine into cAMP [14, 18, 19]. The ability

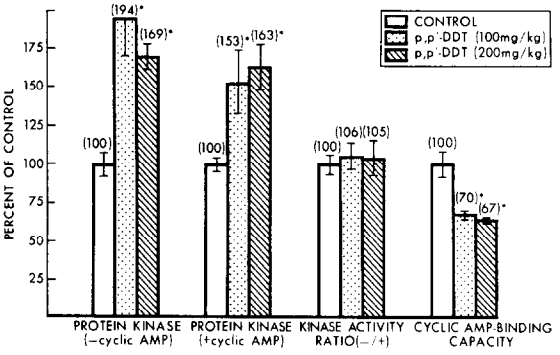

Fig. 3. Effects of *p,p'*-DDT on hepatic protein kinases in crude nuclear preparation. Each bar represents the mean ± S. E. M. of five to six rats in each group. Animals were treated orally with either a 100 or 200 mg/kg dose of *p,p'*-DDT and sacrificed 1 hr later. Data are given in percentages taking the values of control rats as 100 per cent. The asterisks indicate statistically significant differences when compared with the values of control rats ($P < 0.05$).

of exogenous cAMP to mimic the action of DDT and the finding that treatment with theophylline augmented the effects of a submaximal dose of this hydrocarbon on liver and kidney gluconeogenesis support the concept that an initial stimulation of the cAMP–adenylate cyclase system may mediate the observed changes in carbohydrate metabolism [20]. In view of the fact that the diverse effects of cyclic AMP are dependent on the ability of this cyclic nucleotide to stimulate protein kinases [7–13], the findings that pesticide treatment produced a reduction in the cyclic AMP-binding capacity, decreased soluble cyclic AMP-dependent catalytic activity as well as enhanced the kinase activity ratio (−cAMP/+cAMP) provide additional support for the hypothesis that a relationship exists between stimulation of hepatic protein kinases and elevated endogenous cyclic AMP in DDT-treated animals. It is noteworthy that agents such as glucagon and epinephrine, which stimulate the adenylate cyclase–cyclic AMP system of liver, also were found to increase the ratio of protein kinase activity as well as produce a decrease in the ability of this enzyme to bind [^{3}H]cyclic AMP *in vitro* and the activity of cyclic AMP-dependent protein kinase [10, 21]. Recently, Costa *et al.* [22] demonstrated that 3-methylcholanthrene, an agent known to produce proliferation of smooth endoplasmic reticulum and induction of hepatic microsomal enzymes, also activated the cyclic AMP–protein kinase system, suggesting that stimulation of drug-metabolizing enzymes in liver may be mediated through modulation in protein kinase. Since *p,p'*-DDT is also effective in initiating the induction of hepatic microsomal enzymes involved in drug and steroid metabolism and growth of smooth endoplasmic reticulum [23–26], the possibility exists that the activation of the protein kinase system seen in this study might be important in the pesticide-stimulated metabolic responses of liver. The present investigation also indicates that a temporal relationship may exist between the DDT-induced alterations in the hepatic cyclic AMP–adenylate cyclase–protein kinase system and the observed changes in hepatic carbohydrate metabolism. Whereas significant enhancement in the capacity of liver slices to synthesize [^{3}H]cyclic AMP from [^{3}H]adenosine *in vitro* was noted as early as 30 min after administration of the halogenated hydrocarbon and maximal stimulation in protein kinase from both cytosol and crude nuclear fraction occurred at 1 hr, the highest increases in the activities of various gluconeogenic enzymes were seen only between 4 and 5 hr after *p,p'*-DDT treatment (18, 20].

Recent observations indicate that cyclic AMP is involved in the induction of several enzymes in rat liver [27–32]. In addition, it has been shown that treatment with cyclic AMP increases the capacity of liver nuclei to synthesize RNA [33]. Cyclic AMP is known to stimulate the phosphorylation of histones and nuclear acidic proteins in rat liver [12, 13, 34–36], and it has been suggested that the cyclic nucleotide exerts its stimulatory effects at the transcriptional level by stimulating nuclear protein kinases and modifying the interactions between DNA and associated chromosomal proteins [12, 13, 36–39]. Indeed, Martelo and Hirsch [40] observed that a protein kinase from rat liver nuclei stimulated hepatic RNA synthesis and that the stimulatory effect was seen primarily with the nucleolar RNA polymerase. Results from the present study show that 1 hr after the administration of *p,p'*-DDT, protein kinase activity in crude nuclear preparation was markedly elevated (50 per cent), as indicated by an increase in the incorporation of ^{32}P from [γ-^{32}P]ATP into endogenous nuclear substrates. Since the increase in the activities of various hepatic gluconeogenic enzymes observed after treatment with the pesticide was blocked by actinomycin D and cycloheximide [20], it is conceivable that these DDT-induced biochemical alterations may be mediated through an increase in protein kinase activity in the cell nucleus and subsequent enhancement in RNA and protein synthesis. It is also of interest that whereas DDT treatment resulted in a significant decrease in total protein kinase activity of the cytosol (protein kinase activity in presence of cyclic AMP), the activity of the particulate enzyme was markedly elevated. These findings are in accord with the observations of Korenman *et al.* [41] and Jungmann *et al.* [42] showing that, after the activation by cAMP, the soluble protein kinase may be translocated to another cellular compartment where it phosphorylates certain functional proteins to produce the expected physiological responses. Data obtained in this study support the concept that modulation of the cyclic AMP–adenylate cyclase–protein kinase system may be involved in the *p,p'*-DDT-induced alterations in hepatic homeostatic mechanisms of carbohydrate metabolism.

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