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STIMULATION OF RAT LIVER 3-HYDROXY-3-METHYLGLUTARYL-COENZYME A REDUCTASE ACTIVITY BY *o,p'*-DDD

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Abstract—To investigate the mechanism by which *o,p'*-DDD (2,2-bis[2-chlorophenyl-4-chlorophenyl]-1,1-dichloroethane; Mitotane) produces hypercholesterolemia in man, we studied the effect of the drug on hepatic 3-hydroxy-3-methylglutaryl-CoA reductase activity in reverse light-cycled rats. *o,p'*-DDD markedly stimulated reductase activity *in vivo* and *in vitro* in a dose-dependent manner. This effect was not associated with demonstrable adrenocortical toxicity or changes in plasma corticosterone concentrations. Thus, *o,p'*-DDD may elevate circulating cholesterol levels in man by increasing endogenous cholesterol synthesis. In addition, the *o,p'*-DDD-treated rat may serve as a useful model for testing other agents for the ability to suppress endogenous cholesterol synthesis and lower circulating cholesterol levels.

The adrenolytic drug *o,p'*-DDD (2,2-bis[2-chlorophenyl-4-chlorophenyl]-1,1-dichloroethane; Mitotane) has been used in the treatment of hypercortisolism due to adrenal carcinoma [1–3] or Cushing's disease [4–7] as well as in other neoplastic conditions [3]. Common side effects of chronic *o,p'*-DDD therapy include gastrointestinal and neurological complaints and, in men, gynecomastia [8].

Among the most frequent biochemical abnormalities induced by *o,p'*-DDD administration is hypercholesterolemia. Two recent studies [6, 7] document significant elevations of serum cholesterol levels, with or without coincident rises in serum triglycerides, in patients receiving *o,p'*-DDD for treatment of Cushing's disease.

One mechanism by which *o,p'*-DDD may elevate circulating cholesterol levels is by stimulating endogenous cholesterol production. We investigated this possibility in intact rats and isolated rat hepatocytes by examining the effect of *o,p'*-DDD on the activity of microsomal 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase), the rate-limiting enzyme for mammalian cholesterol biosynthesis [9]. We found that *o,p'*-DDD markedly stimulated HMG-CoA reductase activity *in vivo* and *in vitro* in concentrations similar to those administered clinically, and, by this means, the drug may contribute to the rise in serum cholesterol observed in man.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats (Harlan Co., Indianapolis, IN) weighing 230–260 g were used. They were exposed to 12 hr of light (7:00 p.m. to 7:00 a.m.) and 12 hr of darkness (7:00 a.m. to 7:00 p.m.) for at least 10 days prior to use. Throughout these periods of adaptation, the animals had free access to water and formula chow (Purina). Rats used in *in vitro* experiments were killed at 9:00 a.m. and those used for *in vivo* studies at 12 noon. In the *in vivo* studies, *o,p'*-DDD was pulverized, suspended in 3 ml of sterile water, and administered via an orogastric tube to rats lightly anesthetized with ether. Concentrations of 50 mg/kg and 200 mg/kg were employed to reflect the therapeutic dose range for man [6–8]. Control rats received only sterile water. Animals were weighed before each dose. All animals were treated at 4:00 p.m. daily for the 4 days immediately prior to being killed.

Liver cell isolation and incubation

Isolated, morphologically intact, hepatic parenchymal cells were obtained in high yield by modification of the method of Berry and Friend [10]. Rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (Nembutal). Livers were perfused *in situ* with an oxygenated, calcium-free Krebs–Henseleit original Ringer bicarbonate buffer (pH 7.4) containing 0.05% collagenase and gassed with 95% O_2–5% CO_2. The liver was perfused 35–40 min until its consistency was very soft and, then, was excised, gently minced, and shaken in enzyme buffer in an atmosphere of 95% O_2–5% CO_2 for 10 min at 37°. Isolated cells were separated from debris, washed, and resuspended in fresh enzyme-free medium, as described previously [11]. At least 95% of the final cell preparation obtained from each liver consisted of intact parenchymal cells that excluded trypan blue stain. Cells were incubated in 25 ml Erlenmeyer polycarbonate flasks at a concentration of 5×10^8 cells. Crystalline *o,p'*-DDD was suspended in incubation buffer and added in a vol-

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ume of 3.0 ml, at final concentrations of 0.01, 0.1, 1.0 or 10 mM. The total volume per flask was adjusted to 6.0 ml with buffer and the cells were incubated with gentle shaking in a 95% O_2–5% CO_2 atmosphere at 37° for 90 min. The incubation medium contained 0.15 M NaCl, 0.15 M KCl, 0.11 M $CaCl_2$, 0.15 M KH_2PO_4, 0.15 M $MgSO_4 \cdot 7H_2O$, 25 mM $NaHCO_3$ and 1.5% bovine serum albumin (pH 7.4).

Microsomal preparation

From hepatocytes. The method of isolation of microsomes was a modification of that described by Shapiro *et al.* [12]. After incubation, cells were transferred by Pasteur pipette to 15 ml plastic centrifuge tubes and spun at 50 *g* for 2 min. The supernatant fraction was removed and replaced with 1.5 ml of a hypotonic medium (buffer A) containing 1 mM Tris buffer, 10 mM KCl, 10 mM $MgCl_2$ and 18 mM EDTA (pH 7.2). The cells were resuspended, transferred to Dounce homogenizers, and disrupted with 20 strokes. Three and one-half milliliters of a buffer (buffer B) containing 0.25 M NaCl, 50 mM K_2HPO_4, 30 mM EDTA and 1 mM dithiothreitol (pH 7.4) was added to each tube. The suspension was centrifuged twice for 15 min at 12,000 *g* to remove mitochondria and associated HMG-CoA lyase activity and once for 60 min at 100,000 *g*. The supernatant fraction was decanted, and the remaining microsomal pellet was quick-frozen in dry ice–acetone and stored at −70° for up to 3 weeks before assaying.

From whole livers. Chronically treated rats were anesthetized, and their livers were excised and minced, using scalpel blades. Five grams of minced liver in 20 ml of buffer B was homogenized with 4 strokes of a Teflon pestle in a Potter–Elvehjem homogenizer. The homogenate was centrifuged, and the microsomes were isolated, frozen, and stored as described above.

Analyses

HMG-CoA reductase. The assay for HMG-CoA reductase was a modification of that previously described by Edwards and Gould [13]. Microsomal pellets were thawed at 4° and resuspended in buffer B. Protein concentrations were determined using a Coomassie dye-binding method [14]. A microsomal suspension (100 μl) containing approximately 250 mg of protein from isolated cells or 1 mg of protein from whole liver homogenates was added to 12 × 75 mm glass culture tubes (Kimex) and preincubated at 37° for 5 min.* A cofactor–substrate solution (50 ml), consisting of 4.5 mM glucose-6-phosphate (monosodium salt), 450 nmoles NADP, 0.3 I.U. glucose-6-phosphate dehydrogenase and 150,000 dpm DL-hydroxymethyl-[3-^{14}C] glutaryl coenzyme A, was added to the suspension. The samples were incubated at 37° for 15 min and the reaction was terminated by addition of 25 μl of 10 mM HCl.† [^{3}H]Mevalonolactone (10 μl, 30,000 dpm) was added as an internal standard. Samples were incubated for at least 45 min to allow lactonization of the mevalonate, transferred by Pasteur pipette to 400 μl centrifuge tubes (Beckman), and centrifuged for 1 min at 12,000 rpm to sediment denatured protein. A portion of the deprotenized, acidified supernatant fraction was applied to activated chromatogram sheets (Eastman), which were developed in benzene–acetone (1:1) and dried. A mevalonolactone standard was run on each plate. The corresponding sample areas were isolated and counted in 10 ml of modified Bray's fluor. HMG-CoA reductase is expressed as nmoles of [^{14}C]mevalonate formed · (mg microsomal protein)$^{-1}$ · min^{-1}.

Cholesterol and corticosterone. Blood was withdrawn from the inferior vena cava, at the time of sacrifice, into tubes with or without EDTA, centrifuged, and refrigerated. Serum cholesterol was measured by the enzymatic method available from Dow Diagnostics (Indianapolis, IN). Plasma corticosterone was quantitated by radioimmunoassay [15] after separation from other steroids and lipids by a modification of the thin-layer chromatographic method of Kolanski [16] as follows: B21-42 antiserum was developed in rabbits following immunization with corticosterone 21-hemisuccinate-bovine serum albumin conjugate (Endocrine Sciences, Tarzana, CA). Cross reactions of the antiserum with other steroids are given in Table 1. In the purification procedure by thin-layer chromatography, benzene–acetone (2:1, v/v) served as the solvent phase. Merck

Table 1. Steroid cross-reactivity with antiserum B21-42*

Steroid	% Cross-reactivity
Progesterone	57.8
11-Deoxycorticosterone	43.7
20α-Hydroxyprogesterone	16.3
Cortisol	5.2
20β-Hydroxyprogesterone	4.9
11-Desoxycortisol	3.1

* B21-42 antiserum was developed in rabbits following immunization with corticosterone 21-hemisuccinate-bovine serum albumin conjugate. Percent cross-reactivity is given for steroids at 50% bound.

Table 2. R_f values of cross-reactive steroids*

Steroid	R_f
Progesterone	0.76
11-Deoxycorticosterone	0.62
20α-Hydroxyprogesterone	0.61
11-Deoxycortisol	0.50
Corticosterone	0.37

* Steroids were purified by thin-layer chromatography using benzene–acetone (2:1, of v/v) as the solvent phase. Merck SG plates were pre-washed with methanol and were developed twice to 1 cm from the top. The recorded R_f values are those following the second run-up.

* In preliminary experiments, the assay based upon whole liver samples was performed at protein concentrations between 0.1 and 1.2 mg of microsomal protein to establish linearity of the reaction with respect to protein concentration. Based upon these studies, and in accordance with the findings of others [12], 1 mg of microsomal protein was used in subsequent experiments.

† Linearity of mevalonate formation with respect to time was tested at 5, 10, 15, 20, 30 and 45 min of incubation, and established for the time interval of 10–15 min.

SG plates were pre-washed with methanol and were developed twice to 1 cm from the top. Table 2 lists the pertinent R_f values after development of the plates.

History

Adrenal glands were excised, fixed in formalin, and embedded in paraffin. Sections were stained with hematoxylin and eosin and examined by light microscopy.

Chemicals

o,p'-DDD was obtained in crystalline form from NCI (NIH) or as Mitotane (Bristol Laboratories, Syracuse, NY). Radioactive compounds were purchased from the New England Nuclear Corp., Boston, MA. Collagenase was supplied by Worthington Biochemicals, Freehold, NJ, and the Coomassie dye reagent by Bio-Rad Laboratories, Rockville Centre, NY. Other chemicals and enzymes were obtained from the Sigma Chemical Co., St. Louis, MO.

RESULTS

In vivo *studies*

HMG-CoA reductase activity. Figure 1 shows the effect of *o,p'*-DDD on hepatic HMG-CoA reductase activity in rats receiving the drug orally for 4 days. Mevalonate formation in control animals averaged (± S.E.M.) 1.87 ± 0.38 nmoles·(mg protein)$^{-1}$·min^{-1}. Treatment with *o,p'*-DDD stimulated enzyme activity 60%, to 3.00 ± 0.35 nmoles·(mg protein)$^{-1}$·min^{-1}, at a dose of 50 mg/kg and over 4-fold, to 5.71 ± 1.23 nmoles·(mg protein)$^{-1}$·min^{-1}, at a dose of 200 mg/kg.

Cholesterol and corticosterone levels. Serum cholesterol and plasma corticosterone levels were determined in control and treated animals following 4 days of saline or drug treatment. Mean cholesterol levels were slightly, but insignificantly, higher in treated (60 ± 6 mg/dl) compared to control (54 ± 4 mg/dl) animals. The mean corticosterone level in animals receiving 50 mg/kg *o,p'*-DDD was lower (290 ± 40 μg/dl) than in those receiving either no drug (339 ± 44 μg/dl) or 200 mg/kg *o,p'*-DDD (351 ± 42 μg/dl), but the difference did not achieve statistical significance.

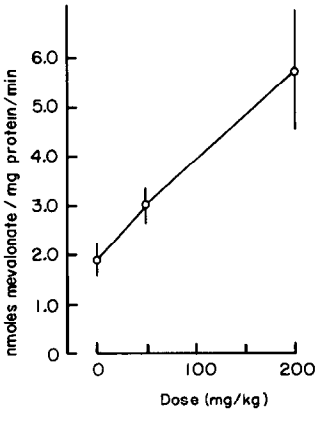

Fig. 1. Effect of chronic *o,p'*-DDD administration on rat liver HMG-CoA reductase activity. Doses of 50 or 200 mg·kg^{-1}·day^{-1} for 4 days were administered by gastric tube to reverse light-cycled rats. The rate of mevalonate formation by hepatic microsomes was determined, as described in the text.

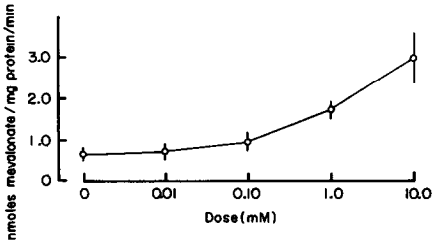

Fig. 2. Effect of *o,p'*-DDD on HMG-CoA reductase activity in isolated hepatocytes. Suspensions of isolated liver cells, obtained from reverse light-cycled rats, were incubated 90 min with or without added *o,p'*-DDD. The rate of mevalonate formation by hepatic microsomes was determined, as described in the text.

Adrenal morphology. Adrenal glands of two animals in each group (control rats, those receiving 50 mg/kg *o,p'*-DDD, and those receiving 200 mg/kg *o,p'*-DDD) were examined grossly and in a blind manner by light microscopy. No discernible morphological differences were apparent among the three groups. Multiple sections of each gland showed preservation of normal adrenocortical structure, including the zona fasciculata.

In vitro *studies*

HMG-CoA reductase activity. To test the direct effect of *o,p'*-DDD on HMG-CoA reductase, suspensions of isolated hepatocytes were incubated with various drug concentrations. Under basal conditions (no added drug), isolated cell microsomes synthesized 0.67 ± 0.05 nmole mevalonate·(mg protein)$^{-1}$·min^{-1} (Fig. 2). At a concentration of 0.01 mM, *o,p'*-DDD had no noteworthy effect on enzyme activity. Within the concentration range of 0.1 to 10 mM, however, *o,p'*-DDD produced a dose-dependent rise in HMG-CoA reductase activity. Mevalonate formation increased 40% above basal, to 0.94 ± 0.16 nmole·(mg protein)$^{-1}$·min^{-1}, with 0.1 mM *o,p*-DDD and increased over 4-fold above basal, to 2.91 ± 0.48 nmoles·(mg protein)$^{-1}$·min^{-1}, at a dose of 10 mM, the highest concentration tested.

Cell viability. As measured by trypan blue exclusion, cell viability ranged between 95 and 98% immediately before incubation and between 88 and 98% immediately following incubation. No effect of *o,p'*-DDD on cell viability was found at any concentration tested.

DISCUSSION

These results show that *o,p'*-DDD markedly stimulates rat liver HMG-CoA reductase activity *in vivo* and *in vitro* in a dose-dependent manner. The doses employed in the chronic feeding studies are similar to those administered clinically to human subjects [6–8]. The rise in serum cholesterol observed

in patients receiving *o,p'*-DDD may thus be due, at least in part, to stimulation of hepatic reductase activity and a consequent increase in endogenous cholesterol production. *o,p'*-DDD is known to stimulate several hepatic microsomal enzyme systems [17] and thus may affect other steps involved in cholesterol biosynthesis. In addition, *o,p'*-DDD may elevate circulating cholesterol by inhibiting lipoprotein cholesterol uptake by peripheral tissues. None of these other possible mechanisms has been examined so far.

Despite an increase in hepatic HMG-CoA reductase activity, *o,p'*-DDD produced no noteworthy change in circulating cholesterol levels. This is not surprising, since, unlike man and several other mammals, the rat is markedly resistant to nutritional or pharmacologic induction of hypercholesterolemia [18]. Hypertriglyceridemia may occasionally accompany the rise in serum cholesterol during *o,p'*-DDD therapy, as reflected in increased very low-density and low-density lipoprotein concentrations [7]. Since newly synthesized cholesterol and triglyceride are released into the circulation only as preformed lipoproteins, the stimulation of cholesterol synthesis by *o,p'*-DDD may necessitate a compensatory rise in endogenous triglyceride production to facilitate lipoprotein formation. Indeed, preliminary experiments in our laboratory have shown that *o,p'*-DDD may stimulate [^{14}C]acetate incorporation into saponifiable lipids in isolated hepatocytes derived from fed rats.

It is reported that glucocorticoids suppress, while adrenalectomy stimulates, cholesterol synthesis and HMG-CoA reductase activity in rat liver [19–24], although the physiologic importance of glucocorticoids in the regulation of hepatic HMG-CoA reductase activity is unknown. Although *o,p'*-DDD is toxic to adrenocortical tissue of man and certain other animals [5, 25–28], the rat is reported to be relatively immune to the adrenolytic effects of the drug [29]. This is consistent with our observations that *o,p'*-DDD did not elicit noteworthy changes in plasma corticosterone levels or adrenal morphology. Thus, the rise in reductase activity observed *in vivo* would not appear to be due to an effect of *o,p'*-DDD on adrenocortical function. This conclusion is further substantiated by our *in vitro* experiments, showing clearly that *o,p'*-DDD is capable of stimulating reductase activity in an isolated cell system without being demonstrably toxic to those cells.

HMG-CoA reductase exists in active (dephosphorylated) and inactive (phosphorylated) forms, the interconversion of which is catalyzed by an ATPMg^{2+}-requiring kinase and Ca^{2+}-requiring phosphatase [18, 30, 31]. It is estimated that, *in vivo*, HMG-CoA reductase exists principally in an inactive form [30] but that its phosphorylation state, as well as its total rate of synthesis may be modulated by various nutritional, hormonal or pharmacological influences. Whether *o,p'*-DDD stimulates reductase activity by affecting the phosphorylation state of the enzyme or by inducing new enzyme synthesis, or both, is unknown. Regardless of its precise site or mechanism of action, *o,p'*-DDD may be a useful tool for studying cholesterol metabolism *in vivo*, specifically to provide a model in which dietary or pharmacological treatments may be tested for their abilities to suppress endogenous cholesterol synthesis and lower circulating cholesterol levels.

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REFERENCES

1. D. M. Bergenstal, R. Hertz and M. B. Lipsett, *Ann. intern. Med.* **53**, 672 (1960).
2. A. M. Hutter, Jr. and D. E. Kayhoe, *Am. J. Med.* **41**, 581 (1966).
3. D. K. Fukushima, H. L. Bradlow and L. Hellman, *J. clin. Endocr. Metab.* **32**, 192 (1971).
4. T. E. Temple, Jr., D. J. Jones, Jr., G. W. Liddle and R. N. Dexter, *New Engl. J. Med.* **281**, 801 (1969).
5. T. Bledsoe, D. P. Island, R. I. Ney and G. W. Liddle, *J. clin. Endocr. Metab.* **24**, 1303 (1964).
6. J. P. Luton, J. A. Mahoudeau, P. H. Bouchard, Ph. Theiblot, M. Hantecouverture, D. Simon, M. H. Laudat, Y. Touitou and H. Bricaire, *New Engl. J. Med.* **300**, 459 (1979).
7. E. D. Schteingart, H. S. Tsau, C. I. Taylor, A. McKenzie, R. Victoria and B. A. Therrien, *Ann. intern. Med.* **92**, 613 (1980).
8. E. M. Gold, *Ann. intern. Med.* **90**, 829 (1979).
9. V. W. Rodwell, J. L. Nordstrom and J. J. Mitschelen, *Adv. Lipid. Res.* **14**, 1 (1976).
10. M. N. Berry and D. S. Friend, *J. Cell Biol.* **43**, 506 (1969).
11. P. W. Stacpoole, *Metabolism* **26**, 107 (1977).
12. D. J. Shapiro, J. L. Nordstrom, J. J. Mitschelen, V. W. Rodwell and R. T. Schimke, *Biochim. biophys. Acta* **370**, 369 (1974).
13. P. A. Edwards and R. G. Gould, *J. biol. Chem.* **249**, 2891 (1974).
14. M. M. Bradford, *Analyt. Biochem.* **72**, 248 (1976).
15. J. Crose and M. Lebel, *Clin. Biochem.* **11**, 32 (1978).
16. J. Kolanski, *J. Steroid Biochem.* **5**, 55 (1974).
17. K. I. Altman, L. L. Miller and C. G. Bly, *Archs Biochem. Biophys.* **31**, 329 (1951).
18. R. W. Mahley, *Atherosclerosis Rev.* **5**, 1 (1979).
19. J. S. Willmer and T. S. Foster, *Can. J. Biochem. Physiol.* **38**, 1393 (1960).
20. N. S. Mejad and I. L. Chaikoff, *Endocrinology* **75**, 396 (1964).
21. P. E. Hickman, B. J. Horton and J. F. Sabine, *J. Lipid Res.* **13**, 17 (1972).
22. C. M. Neprokroeff, M. R. Lakshmanan, G. C. Ness, R. E. Dugan and J. W. Porter, *Archs Biochem. Biophys.* **160**, 387 (1974).
23. M. R. Lackshmanan, R. E. Dugan, C. M. Nepokroeff, G. C. Ness and J. W. Porter, *Archs Biochem. Biophys.* **168**, 89 (1975).
24. R. D. Lillie, M. I. Smith and E. F. Stohlman, *Archs Path.* **43**, 127 (1974).
25. H. B. Haag, J. K. Finnegan, P. S. Larson, M. L. Dreyfuss, R. J. Main and W. Riese, *Ind. Med.* **17**, 477 (1948).
26. A. A. Nelson and G. Woodard, *Archs Path.* **48**, 387 (1949).
27. M. J. Bleiberg and P. S. Larson, *J. Pharmac. exp. Ther.* **21**, 421 (1957).
28. J. H. U. Brown, *Proc. Soc. exp. Biol. Med.* **83**, 59 (1953).
29. Z. H. Beg, J. A. Stonik and H. B. Brewer, Jr., *Proc. natn. Acad. Sci. U.S.A.* **75**, 3678 (1978).
30. J. L. Nordstrom, V. W. Rodwell and J. J. Mitschelen, *J. biol. Chem.* **252**, 8924 (1977).
31. M. S. Brown, J. L. Goldstein and J. M. Dietschy, *J. biol. Chem.* **254**, 5144 (1979).