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Lack of enhanced microsomal enzyme activity by oxandrolone, an inducer of hepatic smooth endoplasmic reticulum

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The relationship of serum cholesterol level to the prevalence of coronary heart disease [1] has stimulated research on plasma lipid-lowering drugs [2]. Oxandrolone (17β-hydroxy-17α-methyl-2-oxa-5α-androstan-3-one, Anavar), a synthetic anabolic steroid [3], effectively lowered plasma triglycerides in several clinical trials [4, 5] and consistently lowered plasma cholesterol in retired breeder male rats [6], an animal model of hyperlipoproteinemia [7]. Recent work suggests that oxandrolone increases the activity of postheparin plasma hepatic lipase, but Ehnholm *et al.* [8] were unable to show that this change was responsible for its lipid-lowering effect.

The finding in this laboratory [9] that oxandrolone increased the surface density of smooth endoplasmic reticulum (SER) in livers of young adult (YA) rats 35 per cent and retired breeder (RB) rats 68 per cent, as measured by quantitative morphometric techniques, is of considerable interest, since this organelle has been implicated in hepatic cholesterol [10] and lipoprotein [11] synthesis. Contrary to the findings of Horvath *et al.* [12], studies in this laboratory have been unable to demonstrate an oxandrolone-induced SER proliferation in rat liver based on qualitative electron microscopy and quantitative biochemical criteria, i.e. total microsomal protein.* Administration of a number of compounds including barbiturates, chlorinated hydrocarbon insecticides, certain steroids, such as prenenolone-16α-carbonitrile and spironolactone, suggests that there is a positive correlation between the amount of SER hypertrophy and the magnitude of microsomal enzyme induction [13–19]. However, Schmucker and Jones [9] suggested that the SER proliferation is a non-specific pharmacological response to oxandrolone.

The present work investigated the significance of this response by measuring alterations in those modalities of SER function most often affected by drugs which induce hypertrophy of this membrane system. The concentration of cytochrome P-450 and cytochrome b_5 and the activities of NADPH-cytochrome *c* reductase and drug metabolism (ethylmorphine *N*-demethylation) in the liver microsomes from YA and RB rats were examined.

Male Sprague–Dawley rats (Holtzman Co., Madison, Wis.) (200–225 g) and recently retired breeder rats (500 g) were housed in individual cages and provided tap water and Purina Laboratory Chow (Purina Ralston Co.) *ad lib.* The rats were fasted for 24 hr prior to collecting tail vein blood from six rats for initial serum lipid determinations. Serum samples were extracted in chloroform methanol (2 : 1) and analyzed for cholesterol according to the method of Rudel and Morris [20] and for triglycerides by the method of Eggstein and Kreutz [21] using the Biochemica Test Combination for Triglycerides (Boehringer Mannheim Corp., Mannheim, West Germany). Oxandrolone was administered orally for 21 days by mixing the drug (100 mg/kg body wt) with 250 mg corn oil margarine (Fleischmann). The RB and YA control animals received 250 mg of the vehicle/day for 21 days. The rats were fasted for 24 hr prior to sacrifice. The animals were killed by decapitation on the morning of day 22. Serum samples were collected and the livers quickly removed, weighed and homogenized in 10 vol. (w/v) of ice-cold 0.25 M sucrose containing 0.05 M potassium phosphate buffer, pH 7.4, using a Potter–Elvehjem homogenizer with a Teflon pestle. Microsomes were prepared as previously described [22], and microsomal protein was determined by the method of Gornall *et al.* [23]. NADPH-cytochrome *c* reductase was measured as described by Masters *et al.* [24] and cytochrome P-450 and cytochrome b_5 were measured by the methods of Omura and Sato [25], using an Aminco Chance spectrophotometer in the split-beam mode. Microsomal *N*-demethylation was measured as previously described [22] using ethylmorphine (6.5 mM) as substrate. Aliquots (1 ml) of the mixture were taken at 1-min intervals for 10 min and mixed with 1 ml of 10% (w/v) trichloroacetic acid. The reaction was linear with time under these conditions. Formaldehyde produced was determined according to Nash [26]. Data were analyzed by Student's *t*-test for differences between the means [27].

The results summarized in Table 1 show that oxandrolone exerted a significant hypocholesterolemic effect on the serum of the RB rats but not on the serum of the YA rats. There was no effect by oxandrolone on serum triglycerides in either the YA or RB rats. The results of the hepatic drug metabolism studies are summarized in Table 2. Liver/body weight ratios in the RB groups were lower (2.5 and 2.6 per cent) than in the YA rats (2.8 per cent), but this result is not statistically significant. Cytochrome P-450 and cytochrome b_5 concentrations in both groups of oxandrolone-treated rats were essentially the same as in the untreated controls. There was no difference in NADPH-cytochrome *c* reductase activity after oxandrolone treatment in either the YA or RB rats. Drug metabolism, determined by the rate of formaldehyde formation

*Unpublished observations.

Table 1. Effect of oxandrolone on serum lipid levels of male young adult and retired breeder rats*

Group	Serum cholesterol (mg/100 ml) Initial	After 21 days of treatment	P†	Serum triglyceride (mg/100 ml) Initial	After 21 days of treatment	P†
Retired breeder	91 ± 9	103 ± 7	NS‡	72 ± 8	74 ± 9	NS
Retired breeder + oxandrolone	86 ± 8	60 ± 6	< 0.02	53 ± 9	45 ± 6	NS
Young adult	74 ± 6	68 ± 2	NS	48 ± 5	41 ± 4	NS
Young adult + oxandrolone	79 ± 4	71 ± 4	NS	50 ± 11	51 ± 5	NS

* All values represent mean ± S. E. M. for six rats.
‡ NS = not significant.
† Difference between initial and post-treatment determinations.

Table 2. Measurements of liver microsomal drug metabolism in young adult and retired breeder rats treated with oxandrolone for 21 days*

	Young adult	Young adult + oxandrolone	Retired breeder	Retired breeder + oxandrolone
Body wt (g)	319 ± 5	311 ± 7	570 ± 22	550 ± 14
Liver wt (g)	9.0 ± 0.1	8.72 ± 0.27	14.09 ± 0.65	14.05 ± 0.71
Liver wt/body wt (%)	2.8 ± 0.1	2.8 ± 0.1	2.5 ± 0.1	2.6 ± 0.1
Cytochrome P-450 (nmoles × mg^{-1} protein)	1.39 ± 0.04	1.43 ± 0.07	1.40 ± 0.05	1.49 ± 0.04
Cytochrome b_5 (nmoles × mg^{-1} protein)	0.55 ± 0.04	0.62 ± 0.05	0.55 ± 0.03	0.64 ± 0.03
NADPH-cytochrome *c* reductase (nmoles × min^{-1} × mg^{-1} protein)	64 ± 6	63 ± 3	60 ± 5	68 ± 7
Ethylmorphine demethylation (nmoles × min^{-1} × mg^{-1} protein)	2.8 ± 0.3	3.6 ± 0.3	2.4 ± 0.1	2.5 ± 0.2

* All values represent mean ± S. E. M. for six to ten rats.

during incubation of microsomes with ethylmorphine, was slightly but not significantly higher in the YA rats compared to the RB rats. Ethylmorphine metabolism was not increased after oxandrolone treatment.

The results of this study confirm and extend previous observations on the effects of oxandrolone on YA and RB rats [6, 9]. The results show that oxandrolone administered for 21 days does not appear to have a marked effect on liver drug metabolism in YA or RB rats. We were unable to demonstrate a significant increase in liver weight/body weight ratios in either YA or RB rats after oxandrolone treatment. Although oxandrolone increased the surface density of SER significantly as measured by quantitative electron microscopy [9], this change does not appear to be accompanied by concomitant increases in microsomal metabolism of ethylmorphine, in the concentrations of cytochrome P-450 and cytochrome b_5, or in the specific activity of NADPH-cytochrome *c* reductase. Some clinical trials have remarked that one parameter of liver function, serum transaminase, may rise after oxandrolone treatment [4, 28] but this report has not been consistent.

In contrast, several other lipid-lowering drugs, such as clofibrate, nafenopin, and bis-(hydroxy-ethyl-thio) 1-10 decane (LL 1558) [29–31], cause marked changes in the liver. Clofibrate, the most successful hypolipidemic drug, causes hepatomegaly in rats [29], hepatic microbody proliferation [30], hypertrophy of the SER [32, 33] and enhanced microsomal metabolism of drugs [34].

It appears that oxandrolone can be added to a growing list of compounds such as D-galactoseamine [35] and dieldrin [36], which, when administered to rats, result in a liver with a marked increase in the amount of SER but normal or reduced activity of drug-metabolizing enzymes. The reason for this lack of association is unclear. Fouts [37] has suggested that prolonged exposure to some inducers (particularly at high doses) may lead to a state where structure and function no longer correlate. Experiments to examine the effects of short-term administration of oxandrolone at lower doses are necessary to resolve this possibility. The present investigation is only a preliminary report based on a single-dose treatment, but the results suggest that oxandrolone is an effective non-toxic lipid-lowering agent meriting further investigation.

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Binding of narcotics and narcotic antagonists to triphosphoinositide

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Triphosphoinositide occurs mainly in the brain as a constituent of membrane. It is both water soluble and lipid soluble and has five negative charges concentrated in a molecule of about 1000 molecular weight. A role for triphosphoinositide in nerve transmission has been considered based on the observation that calcium ion can chelate with triphosphoinositide [1] and that phosphate turnover rate in triphosphoinositide is very rapid [2]. Apart from its physiologic functions, triphosphoinositide may also be involved in opiate action.

It has been reported that morphine administration can deplete brain calcium in animals [3,4] and, since the level of calcium fluctuates with the level of triphosphoinositide [5], there is a possibility that the action of morphine may involve triphosphoinositide. To find any fluctuation in the triphosphoinositide pool during the morphine action may be futile, as the majority of triphosphoinositide is merely a part of myelin structure, and only a small part of total pool size may fluctuate. Mulé [6] studied the turnover of triphosphoinositide and found it to be increased during morphine treatment.

It has been reported that acidic lipids, including triphosphoinositide, can bind with opiates with different affinities in different media. Thus, in the organic phase, morphine is bound more than naloxone, while in the water phase the reverse is true. The differences in binding properties have been related to biologic activity.* This communication reports a further study on the binding of three narcotic agonist–antagonist pairs to triphosphoinositide.

[^{3}H]Naloxone hydrochloride (23.6 Ci/m-mole) was obtained from New England Nuclear, and morphine sulfate from Mallinckrodt. The tartrate salts of levorphanol and [^{3}H]levorphanol (2.4 Ci/m-mole) were donated by Hoffmann-La Roche and naloxone hydrochloride by Endo Laboratories. Triphosphoinositide was extracted from rat brain and purified by the method of Michell *et al.* [7]. Based on the total and individual lipid phosphorus analyses after thin-layer chromatography with potassium oxalate impregnated Silica gel H plates and development with a solvent system of chloroform–methanol–4 N NH_4OH (9:7:2, v/v) [8], the preparation was established to contain no lipids other than phosphoinositide lipids which were present in the amount of 1% monophosphoinositide (MPI), 5% diphosphoinositide (DPI) and 70% triphosphoinositide (TPI). The stability of TPI was examined by the azure assay method [9]; TPI was stable over a year when stored at 25° in chloroform–methanol (19:1, v/v); under neutral pH in water, in heptane, or in octanol, TPI was stable over a month.

Two methods were used to study the binding of narcotic agonists or antagonists in various solvent systems. The binding was quantified by determining the concentration of drug needed to inhibit [^{3}H]levorphanol binding to triphosphoinositide by 50 per cent (ID_{50}).

Organic solvent–water partition. This method consisted of the addition of 1-ml of radioactive drug of varying concentrations in water at pH 6.0 to 1 ml of triphosphoinositide solution in heptane or octanol in a glass tube, 13 × 100 mm, and vortexing the mixture at medium speed for 1 min. After the mixture was centrifuged at 1500 *g* for 10 min, an 0.5-ml aliquot of both phases was removed and the radioactivity was determined by liquid scintillation spectrophotometry with 10 ml of Scinti Verse solution (Fisher Scientific Co.); the counting of an efficiency in this system was determined to about 40 per cent. The amount of drug bound to TPI in the organic phase was calculated by the simplified equation of Weber *et al.* [10]: $M_b = M_t - P \times M_W - M_w$, M_b being the amount of drug bound by TPI in the organic phase, M_t, the total amount of drug used, P, the partition coefficient of drug between the organic phase and the aqueous phase, and M_w, the amount of drug in aqueous phase. The dissociation constants of various TPI–drug complexes were obtained by Scatchard analysis [11]. $M_b/M_f = -M_b/K_d + M_m/K_d$.† M_b being the amount of drug bound, M_f, the amount of free drug, M_m, the maximum amount of drug which could be bound, and K_d, the dissociation constant of the TPI drug complex. The heptane–water partition was used to obtain ID_{50} values of various drugs using 5 μg/ml of triphosphoinositide and 5×10^{-8} *M*[^{3}H]levorphanol and varying concentrations of a test drug.

Equilibrium dialysis. A solution of triphosphoinositide in chloroform–methanol (19:1) was dried *in vacuo* with a rotatory evaporator and the residue was sonicated in water at pH 6.0 to make a 0.01% liposome solution. A coil of dialysis Visking membrane was boiled in 1 mM EDTA solution and then in distilled water and soaked in water at room temperature for several hr. A dialysis cell of 1 ml capacity was assembled with a piece of Visking membrane separating the inner from the outer chamber, each containing a small glass bead for the purpose of agitation. The

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†This equation is obtained by multiplying the original Scatchard equation by the amount of TPI added.