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INFLUENCE OF COMBINED TREATMENT WITH PREGNENOLONE-16α-CARBONITRILE AND SPIRONOLACTONE OR PHENOBARBITAL ON DRUG METABOLISM IN RATS

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Abstract—In female rats, pretreatment with pregnenolone-16α-carbonitrile (PCN), spironolactone or phenobarbital resulted in shorter zoxazolamine and hexobarbital sleeping times and more rapid plasma clearance of zoxazolamine and bishydroxycoumarin. All of these effects were slightly enhanced by simultaneous pretreatment with PCN and spironolactone and greatly increased by concomitant administration of PCN and phenobarbital. However, the observed increases in zoxazolamine and hexobarbital metabolism rates by submitochondrial liver fractions were not further augmented by pretreatment with both of the steroids, while the rates in animals treated with PCN and phenobarbital were only slightly greater than in those which received one of these inducers. It is suggested that PCN and spironolactone share a common receptor and induction mechanism, while the action of phenobarbital is slightly different.

The protection offered by catatoxic steroids, barbiturates and other compounds against intoxication with foreign agents has been shown to result from their action as inducers of liver microsomal enzymes, primarily the NADPH-dependent mixed-function oxidases (cytochrome P-450), which transform lipophilic drugs to more readily excretable derivatives [1–3]. The broad substrate specificity of the cytochrome P-450 enzymes is differentially affected by various types of inducer compounds. Thus, phenobarbital increases the metabolism of a very wide variety of drugs, while 3-methylcholanthrene stimulates benzpyrene hydroxylation but not the biotransformation of hexobarbital or ethylmorphine. The catatoxic steroids, typified by pregnenolone-16α-carbonitrile (PCN) and spironolactone, are similar to phenobarbital in effect, but preferentially enhance ethylmorphine *N*-demethylation relative to hexobarbital or benzpyrene hydroxylation [4–6]. The characteristic differences between phenobarbital and 3-methylcholanthrene have been ascribed partly to the diverse forms of cytochrome P-450 induced [7, 8].

The mechanism by which enzyme induction is initiated is poorly understood. It is now believed that the inducer must initially combine with some receptor molecule, the induction receptor, which is the product of a regulatory gene [9, 10]. The complex thus formed would act as a de-repressor or an inducer to initiate gene expression in the nucleus. In only one case has a macromolecule binding an enzyme inducer been identified: 3-methylcholanthrene is bound to a cytoplasmic protein with properties similar to a glucocorticoid receptor [11]; however, the role of the complex is uncertain. Although the biochemical effects brought about by these inducers are of a wide range and the augmented drug-metabolizing activities are exhibited in increased levels of cytochrome P-450, NADPH-cytochrome *c* reductase, and other proteins, as well as in an enhanced electron flux and heightened substrate binding to cytochrome P-450, it should be appreciated that all these changes result from the initial interaction of the inducer with the macromolecular receptor. The function of such receptors may be studied *in vivo* by judicious selection of inducing agents, given singly or in combinations, and by measuring their effects on drug-metabolizing enzyme activities.

Considering the versatility of catatoxic steroids in inducing a wide range of enzyme activities and their potential clinical applications [12], it seemed of interest to explore the interrelation of their inductive effects and to learn whether simultaneous administration of two catatoxic compounds could improve protection against a series of toxicants. Thus, we set out to determine the quantitative effects of PCN and spironolactone and also their interaction with phenobarbital in inducing liver drug-metabolizing enzymes, aiming to elucidate the initial step of the induction phenomenon at the molecular level.

MATERIALS AND METHODS

All experiments were performed on female Charles River CD rats (Canadian Breeding Farms and Laboratories Ltd., St. Constant, Quebec), with a mean initial body weight of 100 g (range 95–105 g). Maintained *ad lib.* on Purina laboratory chow and tap water, these animals were pretreated with inducer compounds twice daily for 3 days and were given the test drugs, and their livers excised 18 hr after the last treatment. PCN (Upjohn, 20 μmoles), spironolactone (Searle, 20 μmoles), pregnenolone (Schering, 4 or

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Table 1. Effect of microsomal enzyme inducers on the response to zoxazolamine*

Conditioner I (dose)	Paralysis time (min)	Conditioner II (dose)	Paralysis time (min)	Combined I + II paralysis time (min)
H_2O	244 ± 13	Peanut oil	219 ± 13	
PCN (0.2 μmole)	231 ± 18	Spironolactone (20 μmoles)	69 ± 3	82 ± 7†
PCN (0.4 μmole)	140 ± 11	Spironolactone (20 μmoles)	69 ± 3	72 ± 6†
PCN (2 μmoles)	57 ± 3	Spironolactone (20 μmoles)	69 ± 3	46 ± 3‡
PCN (2 μmoles)	57 ± 3	Phenobarbital (20 μmoles)	57 ± 4	38 ± 3§
Spironolactone (20 μmoles)	69 ± 3	Phenobarbital (20 μmoles)	57 ± 4	50 ± 3†
Pregnenolone (30 μmoles)	203 ± 27	PCN (2 μmoles)	57 ± 3	45 ± 2§
Pregnenolone (4 μmoles)	210 ± 14	PCN (2 μmoles)	57 ± 3	58 ± 6†
Pregnenolone (4 μmoles)	210 ± 14	Spironolactone (20 μmoles)	69 ± 3	70 ± 4†

* Zoxazolamine (10 mg/100 g body weight) was injected i.p. Each value is the average, with standard error, for 6–25 animals.

† $P < 0.005$ compared to conditioner I, not significant compared to conditioner II.

‡ $P < 0.01$ compared to conditioner I, $P < 0.005$ compared to conditioner II.

§ $P < 0.005$ compared to conditioner I, $P < 0.005$ compared to conditioner II.

30 μmoles) or phenobarbital sodium (Allen & Hanbury, 20 μmoles) was given p.o. in 1 ml of distilled water, the steroids as micronized suspensions containing 0.1% Tween 80. Lower doses of PCN were injected i.p. as a solution in 0.5 ml peanut oil (Planter's), in order to minimize interactions of the inducers in absorption, considering that administration of PCN alone by this route gave an effect identical to that observed after treatment p.o. in water. In cases where two compounds were given p.o., the treatments were at least 1 hr apart.

Satisfactory control experiments were conducted with each group of tests; measurements on rats which received two inducers were performed alongside those on animals which were given either one of the inducers or water. All experimental values are reported as averages with standard error of the mean.

Zoxazolamine (K & K Laboratories, 10 mg/100 g body weight in 1 ml of distilled water) was given i.p., the paralysis time being determined as the period between the loss and return of the righting reflex. Blood (3 ml) was taken by aortic puncture, after light ether anesthesia, into a heparinized syringe. Urine was collected after ligature of the urethra and excision of the bladder 2 hr later. Zoxazolamine concentrations in plasma and urine were analyzed by the method of Burns *et al.* [13]. Drug-free plasma and urine from conditioned and non-conditioned animals were used for preparation of standards and blanks.

Bishydroxycoumarin [Abbott, 5 mg/100 g body weight, dissolved in 0.5 ml of distilled water containing 50 mg Tris(hydroxymethyl)aminomethane; solution pH 9.2] was administered i.v. under light ether anesthesia. Blood (3 ml) was taken 20 hr later by aortic puncture into a syringe containing 0.1 M sodium oxalate (0.3 ml). Prothrombin time was measured with an Emdeco prothrombin timer using Dade thromboplastin reagent. Bishydroxycoumarin concentrations in plasma were analyzed by the method of Axelrod *et al.* [14].

Hexobarbital sodium (Sterling-Winthrop, 10 mg/100 g body weight in 1 ml of distilled water) was given i.p.; sleeping time was assessed as for zoxazolamine paralysis.

Livers were excised, 9000 *g* supernatant fractions were prepared and the metabolism rates *in vitro* of zoxazolamine were measured as described by Szabo *et al.* [15], the quantity of unchanged zoxazolamine being determined by the method of Conney *et al.* [16]. The rate of metabolism of hexobarbital by the 9000 *g* fraction was assessed by a procedure identical to that for zoxazolamine, except that the incubation time was 30 min and the reaction was stopped by the addition of 2 ml of 0.8 M phosphate buffer, pH 7.0. Unchanged hexobarbital was analyzed by extraction into petroleum ether, followed by re-extraction into 0.8 M phosphate buffer (pH 11.0) and reading the absorbance at 245 nm [17].

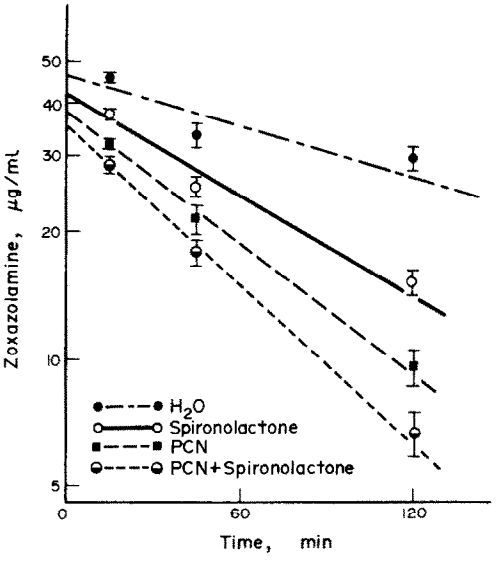

Fig. 1. Effect of inducer pretreatment on plasma clearance of zoxazolamine. Rats were treated with PCN (2 μmoles, i.p.), spironolactone (20 μmoles, p.o.), or a combination of both steroids, twice daily for 3 days. Each point represents the average value for eight to ten animals, with standard error. For the slope of the regression line for PCN + spironolactone, the probability is <0.05 compared to PCN, and <0.005 compared to spironolactone.

Table 2. Effect of PCN and spironolactone on urinary excretion of zoxazolamine*

Conditioner (dose)	Urine volume (ml)	Zoxazolamine concn (μg/ml)	Total zoxazolamine excreted (μg)
H_2O	0.33 ± 0.07	26.3 ± 4.9	8.1 ± 1.3
PCN (2 μmoles)	0.65 ± 0.15	10.0 ± 3.5	5.0 ± 1.0
Spironolactone (20 μmoles)	0.38 ± 0.03	22.1 ± 4.9	7.8 ± 1.5
PCN (2 μmoles) + spironolactone (20 μmoles)	0.67 ± 0.10	5.0 ± 2.0	2.5 ± 0.6

* Urine was collected for 2 hr after administration of zoxazolamine. Each value is the average for six animals.

Each group of data was evaluated by one-way analysis of variance [18, 19]. In every case, the hypothesis that differences (among slopes of regression lines in Fig. 1) were due to random error was rejected with $P < 0.005$. The significance of the observed differences between pairs of (mean) values or slopes was determined by Student's *t*-test, using pooled variance in the case of homogeneous variance (see Tables 3 and 4, Fig. 1), and individual variances in the case of heterogeneous variance (see Tables 1 and 5).

Table 3. Effect of microsomal enzyme inducers on the response to hexobarbital*

Conditioner (dose)	Sleeping time (min)
H_2O	78 ± 4
PCN (2 μmoles)	32 ± 3
Spironolactone (20 μmoles)	36 ± 2
PCN + spironolactone	24 ± 2†
Phenobarbital (20 μmoles)	10 ± 1
PCN + phenobarbital	0 ± 0‡

* Hexobarbital (10 mg/100 g body weight) was injected i.p. Each value is the average, with standard error, for eight animals.

† $P < 0.05$ compared to PCN alone, $P < 0.005$ compared to spironolactone.

‡ $P < 0.005$ compared to PCN or phenobarbital alone.

RESULTS

Pretreatment with PCN, spironolactone or phenobarbital considerably reduced zoxazolamine paralysis time, as shown in Table 1. Furthermore, simultaneous administration of PCN (2 μmoles) and spironolactone (20 μmoles) produced a small but significant decrease in the sleeping time beyond that induced by either compound alone. The variations in the plasma levels of zoxazolamine were consistent with the observed paralysis times [20], the drug concentrations being considerably diminished in rats pretreated with PCN or spironolactone and reduced further in those given both steroids (Fig. 1). The quantity of zoxazolamine excreted in the urine of pretreated animals in 2 hr was roughly parallel to the plasma levels (Table 2). When the dosage of PCN was decreased to 0.2 or

Table 4. Effect of microsomal enzyme inducers on metabolic rates *in vitro* of zoxazolamine and hexobarbital*

Conditioner (dose)	% Liver	Zoxazolamine (μmoles/g liver/hr)	Hexobarbital (μmoles/g liver/hr)
H_2O	4.64 ± 0.09	2.46 ± 0.12	2.28 ± 0.20
PCN (2 μmoles)	5.60 ± 0.06	5.71 ± 0.20 (232)	3.37 ± 0.21 (148)
Spironolactone (20 μmoles)	5.02 ± 0.09	4.34 ± 0.14 (176)	5.12 ± 0.27 (225)
PCN (2 μmoles) + spironolactone (20 μmoles)	5.52 ± 0.06	5.61 ± 0.14† (228)	4.99 ± 0.39‡ (219)
H_2O	4.75 ± 0.13	1.79 ± 0.06	
PCN (0.4 μmole)	4.80 ± 0.08	2.91 ± 0.22 (163)	
Spironolactone (20 μmoles)	5.19 ± 0.27	4.42 ± 0.23 (247)	
PCN (0.4 μmole) + spironolactone (20 μmoles)	5.44 ± 0.13	4.53 ± 0.09‡ (253)	
H_2O	4.56 ± 0.09	2.19 ± 0.17	2.28 ± 0.20
PCN (2 μmoles)	5.71 ± 0.09	5.08 ± 0.22 (232)	3.37 ± 0.21 (148)
Phenobarbital (20 μmoles)	5.90 ± 0.15	4.90 ± 0.19 (224)	13.01 ± 0.50 (570)
PCN (2 μmoles) + phenobarbital (20 μmoles)	6.46 ± 0.11	5.71 ± 0.09§ (261)	13.31 ± 0.18‖ (584)

* The 9000 *g* supernatant fractions from liver homogenates were incubated at 37° with 0.4 μM substrate in a medium (final volume 3 ml) containing Tris–HCl buffer (pH 7.4), $MgCl_2$ (5 mM), glucose 6-phosphate (5 mM) and NADP (0.4 mM). All values are averages for seven to ten animals. Figures in parentheses indicate percentage of control.

† Not significant compared to PCN alone, $P < 0.005$ compared to spironolactone alone.

‡ Not significant compared to spironolactone alone, $P < 0.005$ compared to PCN alone.

§ $P < 0.05$ compared to PCN alone, $P < 0.005$ compared to phenobarbital alone.

‖ Not significant compared to phenobarbital alone, $P < 0.005$ compared to PCN alone.

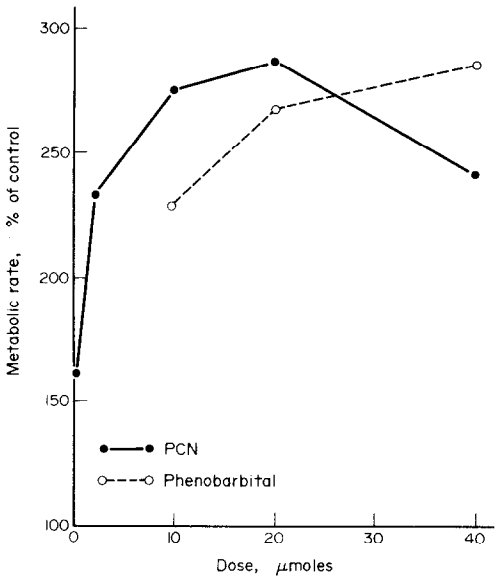

Fig. 2. Effect of pretreatment with PCN or phenobarbital on zoxazolamine metabolic rate in the 9000 *g* liver supernatant fraction. Each point represents the average value for six to ten animals.

0.4 μmole, the animals treated with the two steroids were not paralyzed for a shorter period than those which received spironolactone alone.

On the other hand, simultaneous administration of phenobarbital (20 μmoles) and PCN (2 μmoles) exerted an additive action in reducing the paralysis time more effectively than either compound separately. Pregnenolone, a very weak inducer of liver microsomal drug-metabolizing enzymes [21], had no significant influence on zoxazolamine paralysis time; in PCN-treated animals, a slight enhancement of the protective action was observed with 30 μmoles pregnenolone.

In a similar way, hexobarbital sleeping time was further decreased after combined pretreatment with PCN and spironolactone, while conjoint administration of the cyanosteroid with phenobarbital gave markedly improved protection (Table 3).

Zoxazolamine metabolism by the submitochondrial supernatant fraction of liver homogenate *in vitro* was also strikingly enhanced by pretreatment with PCN, spironolactone or phenobarbital (Table 4). The alterations in enzyme activity (expressed as a percentage of controls) with varying doses of PCN or phenobarbital are shown in Fig. 2. The liver fractions from rats pretreated with both compounds did not metabolize the drug at a higher rate than those which received only one steroid. Similar results were obtained using dosages of PCN that induced an effect larger or smaller than that of spironolactone. Furthermore, the pattern of hexobarbital metabolism rates from similarly pretreated rats was entirely analogous (see Table 4).

In contrast to the results just described, combined treatment with PCN and phenobarbital produced significant, though small, additive effects on the rate of zoxazolamine metabolism *in vitro*.

Analogously, pretreatment with PCN, spironolactone or phenobarbital accelerated the elimination of bishydroxycoumarin from plasma, as shown both by the reduced prothrombin times and the decreased drug concentrations (Table 5). Parallel to the results obtained for zoxazolamine, an additive effect was observed when either PCN and spironolactone or PCN and phenobarbital were given together.

DISCUSSION

Considering that steroid receptor complexes are frequently characterized by extremely tight binding and that liver microsomal enzyme inducers often have a long duration of action, it was envisaged that a dosage of inducer sufficient to produce a maximal response might well saturate the hypothesized receptor sites and preclude any additional response due to the simultaneous administration of another inducer compound. Thus, in each experiment, the dosage of inducer selected was generally one which elicited a near-maximal response, in terms of counteracting the effects of each particular drug. Since PCN is a powerful inducer of zoxazolamine hydroxylase activity, a rather low dosage (2 μmoles) was initially chosen, in consideration of Fig. 2 and earlier reports [22]. Spironolactone is less potent than PCN[1]; consequently, a higher dose (20 μmoles) was used. The

Table 5. Effect of microsomal enzyme inducers on plasma levels of bishydroxycoumarin and prothrombin time*

Conditioner	Bishydroxycoumarin conen (μg/ml plasma)	Prothrombin time (sec)
H_2O	11.9 ± 2.2	23.7 ± 4.0
PCN	1.9 ± 0.3	12.7 ± 0.3§
Spironolactone	2.3 ± 0.4	14.3 ± 1.2§
PCN + spironolactone	0.7 ± 0.2†	12.7 ± 0.2§
Phenobarbital	0.4 ± 0.1	12.2 ± 0§
PCN + phenobarbital	0.1 ± 0.05‡	12.2 ± 0§

* Each conditioner was given at a dosage of 20 μmoles. Plasma was collected 20 hr after i.v. administration of bishydroxycoumarin (5 mg/100 g body weight). Each value is the average for six to nine animals.

† $P < 0.01$ compared to PCN or spironolactone alone.

‡ $P < 0.05$ compared to phenobarbital alone, $P < 0.005$ compared to PCN.

§ $P < 0.05$ compared to H_2O.

working dosage of phenobarbital (20 μmoles) was chosen in a similar manner (see Fig. 2). In the case of bishydroxycoumarin metabolism, it had been reported that PCN is a poor [20] and spironolactone a moderate inducer [23], and so equimolar doses of 20 μmoles were given.

The enhanced effect of conjoint treatment with PCN (2 μmoles) and spironolactone (20 μmoles), in decreasing zoxazolamine paralysis time and accelerating drug clearance from plasma relative to treatment with either steroid alone, was not explained by an augmented induction of liver microsomal zoxazolamine hydroxylase activity—the metabolic rate *in vitro* was identical to that evoked by PCN alone. Nor did this effect result from increased urinary excretion of the unchanged drug. The biliary clearance of zoxazolamine, which was found to be heightened by spironolactone [24], may contribute to the decreased drug level. Alternatively, the phenomenon may arise from differential induction of zoxazolamine hydroxylase activity in extra-hepatic tissues, such as the lung, kidney or intestine.

Analogous results were obtained for the metabolism of other drugs: the pattern for hexobarbital metabolism, both *in vivo* and *in vitro*, paralleled that of zoxazolamine, and the induced plasma clearance of bishydroxycoumarin showed the same behavior. Thus, the effect of an inducer on liver microsomal enzymes is neither increased nor decreased by the presence of a second, less potent inducer, suggesting the possibility that these two steroids compete for the same receptor site in the initial step of the induction process, and that binding in such an inducer–receptor complex may be fairly tight. Such competition is strongly reminiscent of the behavior of other pairs of related enzyme inducers. Simultaneous administration of maximal doses of the carcinogenic polycyclic hydrocarbons 3-methylcholanthrene and benzpyrene does not result in stimulation of enzyme activity beyond that found for either compound alone [25, 26]. These compounds induce the same type of cytochrome P-450 and most likely function via the same mechanism, initially binding to the identical receptor. The same result has been observed for phenobarbital and chlordane [27], which, although not closely related chemically, show similar induction patterns, and for the closely related compounds norepinephrine and isoproterenol in experiments with hepatocytes in culture [28].

In contrast, there have been numerous reports in which treatment with two inducer compounds of different type elicited an additive stimulation of enzyme activity [25, 26, 28–30]. In the classic combination of phenobarbital and 3-methylcholanthrene, the compounds are structurally dissimilar, show a different binding behavior with cytochrome P-450, and induce diverse forms of cytochrome P-450 hemoprotein with widely different enzyme activities; conjoint administration gives enzyme activities that are strictly additive. Thus, it is concluded that the compounds function via independent mechanisms.

Why, then, is additive stimulation not observed for pairs of dissimilar inducers in general, and for the combination of PCN and phenobarbital in particular? The properties of the system induced by each of these compounds are qualitatively similar, yet significant differences exist among the resultant enzyme activities [4, 5]. The data on combined pretreatment, both *in vivo* and *in vitro*, suggest that the cyanosteroid and the barbiturate exhibit degrees of both additive and competitive effects on the induction mechanism. One cannot rule out the possibility that the two compounds interact with the same induction receptor; the complexes thus formed would not have the same properties and might affect the genetic machinery of the cell differently, resulting in preferential induction of different forms of cytochrome P-450 or of other links in the oxidation chain. On the other hand, the two inducers may have distinctive receptors, but the two types of drug–receptor complexes, inducing the synthesis of similar proteins, may compete at the nuclear level.

We have been unable to demonstrate antagonism against the inductive effect of PCN by pregnenolone, a steroid with a very similar chemical structure, yet with no action of its own. Thus, the receptor for the cyanosteroid is fairly specific in its binding properties, and the inductive capacity of a compound seems to be determined more by its capability to bind to this receptor than by the ability of the resultant complex to perform its inductive function.

One is thus left with the conclusion that PCN and spironolactone appear to act via an identical mechanism and compete for the same specific receptor site, but that distinctive pathways may exist for different types of inducers, such as phenobarbital.

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