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Comparison between cobalt and pyrazole in the increased expression of coumarin 7-hydroxylase in mouse liver

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The multigene family of heme-containing P450 isozymes catalyses the metabolism of a multitude of exogenous and endogenous compounds [1]. Heavy metals usually decrease the ability of the P450 system to metabolize xenobiotics by decreasing the P450 associated activities [2]. In contrast to this general rule, it has been reported that cobalt selectively increases the metabolism of coumarin and its derivatives in mouse liver [3, 4].

We have shown previously that pyrazole is an efficient and selective inducer of coumarin 7-hydroxylase (COH) [5]. The P450 isozyme supporting the activity of COH, designated as P450Coh, has recently been purified to homogeneity [6] and a specific antibody has been raised against it [7]. On the other hand, Negishi and co-workers [8–10] purified, cloned and sequenced two closely related P450-dependent testosterone 15α-hydroxylases, Type I and Type II $P450_{15\alpha}$. Type I $P450_{15\alpha}$ supports testosterone 15α-hydroxylation, while type II $P450_{15\alpha}$ has been shown to be identical with P450Coh [8, 11, 12].

The availability of antibody and cDNA probes for the $P450_{15\alpha}$/P450Coh complex has made it possible to study the effect of heavy metals on the expression of this P450 system. We report here that treatment of mice with cobalt induces the activities linked with P450Coh and that this is accompanied by accumulation of $P450_{15\alpha}$/P450Coh mRNA and protein. However, significant, strain dependent, differences seemed to exist between the effects of cobalt and pyrazole.

Materials and Methods

Chemicals. Cobaltous chloride ($CoCl_2 \cdot 6H_2O$) was purchased from Merck (Darmstadt, F.R.G.). Coumarin, 7-hydroxycoumarin and pyrazole were from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Nucleic acid grade agarose, DNA and RNA molecular weight markers were from Pharmacia (Uppsala, Sweden). Guanidine thiocyanate was from Fluka (Buchs, Switzerland). [τ-^{32}P]dCTP (3000 Ci/mmol) was obtained from NEN-Du Pont (Wilmington, DE, U.S.A.).

Treatment of animals. Adult male C57BL/6N and DBA/2N mice (mean weight about 25 g) were used throughout the study. Cobalt was dissolved in saline and injected subcutaneously as a single daily dose of 125 μmol/kg for 3 consecutive days. As a reference inducer, pyrazole was injected i.p. at a dose of 200 mg/kg (2.9 mmol/kg) once daily for 3 consecutive days [13]. The mice were fasted overnight before killing.

Preparation of microsomes and monooxygenase assays. Livers from 3–5 mice were divided into two parts and the microsomal fraction was prepared by differential centrifugation from the first portions. Microsomal protein content was determined by the method of Bradford [14]. Cytochrome P450 content was determined as originally described by Omura and Sato [15]. COH activity was measured by the method of Aitio [16] using 100 μM coumarin as substrate. Testosterone 15α-hydroxylase activity was determined according to Waxman *et al.* [17].

Western immunoblotting. The preparation and validation of anti-P450Coh antibody has been reported earlier [7]. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis and immunoblot conditions were as described [18]. For P450Coh protein quantitation, a standard curve was prepared with 0.1–1 pmol of purified P450Coh, followed by measuring the staining intensities with a Macbeth TR944 Transmission Reflection densitometer (Kollmorgen Corp., Newburgh, NY, U.S.A.).

Preparation of cDNA probes. The cloning of cDNA p15α-15 coding for type II $P450_{15\alpha}$ has been described previously [9, 10]. The P15α-15 was inserted into pUC9 plasmid and transformed into *Escherichia coli* MC1051.

Table 1. Summary of the magnitudes of increases in enzyme catalytic activity, protein level and mRNA level

Strain	Treatment	COH	Testosterone 15α-hydroxylase	P450Coh protein	$P450_{15\alpha}$ mRNA
DBA/2	Control	0.11 ± 0.03 (1.0)	0.12 ± 0.02 (1.0)	1.0	1.0
	Cobalt	0.28 ± 0.05† (2.5)	0.15 ± 0.03 (1.3)	1.4	1.7
	Pyrazole	0.78 ± 0.13‡ (7.1)	0.25 ± 0.08* (2.1)	3.6	20.5
C57BL/6	Control	0.03 ± 0.01 (1.0)	0.15 ± 0.04 (1.0)	1.0	1.0
	Cobalt	0.13 ± 0.03‡ (4.1)	0.24 ± 0.05* (1.6)	3.2	3.0
	Pyrazole	0.18 ± 0.03‡ (5.9)	0.28 ± 0.10* (1.9)	4.0	1.6

The data is derived from an experiment in which groups of three to five mice were treated with three consecutive daily injections of cobalt chloride (125 μmol/kg) or pyrazole (200 mg/kg). The assays of enzyme activities were done in individual, and immunoblot and RNA slot-blot analysis in pooled samples as described in the text. The enzyme activities are expressed in nmol/min × mg microsomal protein ± SD. The numbers in parentheses in connection with enzyme activities and those for immunoblots and slot-blots denote fold increase relative to the control value.

Significance of the differences: *$P < 0.05$; †$P < 0.005$; ‡$P < 0.001$.

Plasmid linearized with HindII was used as a probe. As a control probe, human actin cDNA [19] was used. cDNA probes were labeled with α-^{32}P-CTP to specific activity of about 10^9 cpm/μg DNA [20].

Preparation of RNA and slot blots. Total RNA was isolated from the pooled second portions of the livers by the guanidine thiocyanate method of Chirgwin *et al.* [21]. The purity of RNA samples was controlled by their UV absorbance and agarose gel electrophoresis [22]. Northern blots were performed in 2.2 M formamide containing agarose gels according to Ref. 22. Slot-blots were done with a Minifold II apparatus (Schleicher & Schuell, Dassel, F.R.G.). The intensity of bands was quantitated with the MacBeth TR944 densitometer. The results were normalized to actin mRNA expression.

Statistics. Kruskal–Wallis analysis of variance was used for statistical calculations [23].

Results and Discussion

After preliminary studies we decided to give cobalt once daily for 3 consecutive days at a dose of 125 μmol/kg (250 μmol/kg was overtly toxic). Repeated treatment of DBA/2 mice with 125 μmol/kg was clearly more effective in inducing COH than a single administration, whereas in C57BL/6 mice, repeated and single administrations evoked a similar response. The studies are summarized in Table 1. The increase in the amount of immunodetectable P450Coh correlated fairly well with the increase in the catalytic activity of COH, although in DBA/2 mice the increase in catalytic activity was somewhat stronger than in the amount of protein.

To elucidate whether cobalt and pyrazole affect also Type I $P450_{15\alpha}$, testosterone 15α-hydroxylase activity was assayed. In DBA/2 mice, cobalt caused no marked changes in this activity, whereas pyrazole treatment increased it 2.2-fold. In C57BL/mice, both cobalt and pyrazole elicited a 1.8-fold increase in the activity of liver testosterone 15α-hydroxylase. In every case, the increase in testosterone 15α-hydroxylase activity was considerably lower than the increase in COH activity.

Figure 1A shows a Northern blot of total RNA from DBA/2 livers. A strong 2.1-kb band is detected after pyrazole treatment. The signal was much weaker with

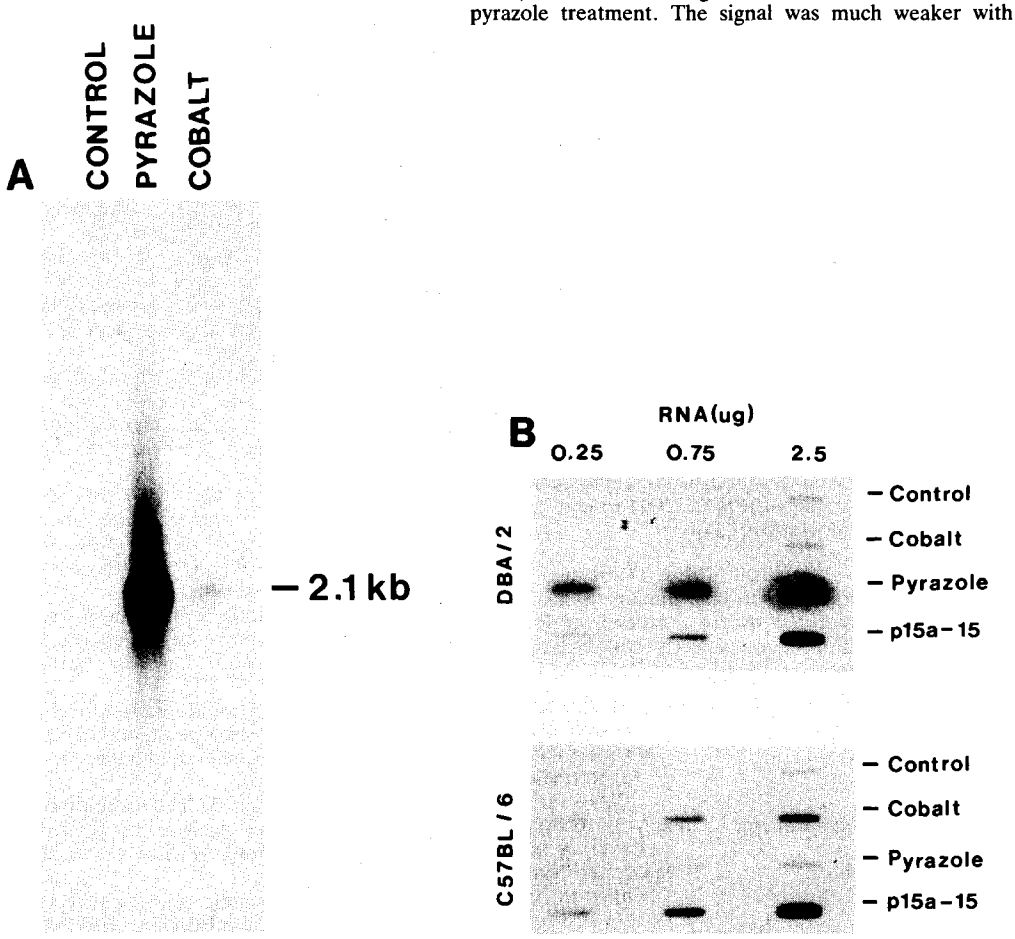

Fig. 1. (A) Northern blot hybridization analysis of total RNA extracted from DBA/2 mouse liver. Twenty-microgram aliquots of RNA were electrophoresed in a formaldehyde-denatured agarose gel, transferred to Hybond-C Extra filters, hydridized with [^{32}P]p15α-15 cDNA and analysed by autoradiography. (B) Slot-blot hybridization analysis of RNA extracted from livers of DBA/2 and C57BL/6 mice. Total cellular RNA from each sample was spotted onto Hybond-C Extra filter in three different concentrations. p15α-15 cDNA was applied to the bottom row at concentrations of 1–16 pg. The filter was hybridized with [^{32}P]p15α-15 cDNA and the signals were quantitated as described in Materials and Methods.

RNA derived from cobalt-treated mice, and almost negligible in case of control animals. A similar size signal was observed in treated C57BL/6 mouse RNA (not shown).

Expression of p15α-15-hybridizable mRNA was quantitated by slot-blot analysis, which demonstrated the increase of the amount of hepatic P450Coh mRNA following cobalt and pyrazole treatment (Fig. 1B). When data are normalized for RNA loading using actin cDNA, the level of induction in case of DBA/2 mice is 1.7-fold after cobalt and at least 21-fold after pyrazole treatment. In C57BL/6 mice, the increase was 3-fold after cobalt and 1.6-fold after pyrazole treatment (Table 1).

The cDNA probe (p15α-15) and antibody (anti-P450Coh) used in this study do not distinguish between the highly homologous Type I and Type II $P450_{15\alpha}$s. There are, however, several reasons to believe that the Type II gene is primarily affected by cobalt and pyrazole. First, diagnostic restriction endonuclease digestion analysis [10] shows that pyrazole causes only a modest increase in Type I mRNA in male mice while potently increasing Type II mRNA expression. Second, testosterone 15α-hydroxylase, the activity catalysed by Type I $P450_{15\alpha}$, is increased only moderately by both cobalt and pyrazole (see Table 1). Therefore, only a minor part of the increased expression of P450Coh/$P450_{15\alpha}$ by cobalt and pyrazole can be ascribed to the Type I $P450_{15\alpha}$

Previous studies [4–6] suggest that the inductive properties of cobalt and pyrazole are similar. However, present results suggest several important differences between the two compounds. First, pyrazole, but no cobalt, decreases many other monooxygenase activities and total P450 content [6, 13]. Second, pyrazole is more potent than cobalt, particularly in DBA/2 mice. Third, increase of mRNA by pyrazole, but not by cobalt, is highly strain dependent.

In summary, the data in this report show that administration of both cobalt and pyrazole results in an elevation in the amount of hepatic mRNA encoding for microsomal P45015α/P450Coh, an increase in the amount of P450Coh protein, and an activation of COH and to a lesser extent testosterone 15α-hydroxylase in two inbred strains of mice. Considerable quantitative differences between the two compounds and the two mouse strains in the response suggest that the effects of cobalt and pyrazole are mediated, at least partly, through different mechanisms. It is of interest that human hepatic COH resembles very closely that in the mouse liver [24].

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Structure–activity relationships for various *N*-alkylcarbamyl esters of choline with selective nicotinic cholinergic properties

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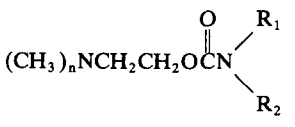
In the course of investigating the structure–activity relationships of various carbamate esters of alkylamino alcohols, it was observed that the addition of a methyl substituent to the carbamyl N of carbamylcholine increases the nicotinic cholinergic properties of carbamylcholine, while virtually abolishing its muscarinic properties [1]. This observation has led to the development of [^{3}H]methylcarbamylcholine ([^{3}H]MCC) as a prototypic ligand for investigating the nicotinic recognition site in brain tissue [1–4]. Since the addition of an aromatic or cycloalkyl substituent on the carbamyl N imparted antagonistic properties to carbamylcholine, a study was undertaken to determine the effect of varying alkyl chain lengths on the nicotinic properties of newly synthesized N-substituted carbamate esters of choline. The compounds were evaluated for their ability to compete for [^{3}H]MCC, [^{3}H]nicotine, and [^{3}H]quinuclidinylbenzilate ([^{3}H]QNB) binding and for their ability to produce prostration in rodents.

Methods

Synthesis of dimethylaminoethyl (DMAE) dimethylcarbamate and dimethylcarbamylcholine (DMCC). The compounds synthesized and evaluated in the present study had the general chemical structure:



where R_1 = H (except for one case where it was methyl); R_2 = methyl, ethyl, propyl, butyl, or phenyl; and n = 2 or 3.

To 0.06 mol of dimethylaminoethanol in 50 mL of dry toluene was added 0.03 mol of dimethylcarbamyl chloride. The solution was refluxed gently for 3 hr during which time a light brown oil formed. After cooling overnight at room temperature, the oil (bottom layer) solidified. The product (DMAE dimethylcarbamate) was recovered from the liquid phase by first evaporating half of the solvent and removing by filtration the residual dimethylaminoethanol hydrochloride. A pale yellow oil (85% yield) was obtained when the filtrate was evaporated to dryness *in vacuo*.

An analysis of DMAE dimethylcarbamate by infrared and NMR spectroscopy yielded the following:

i.r. bands 1710, 1200, 1400, 1460, 1385, 1500, (1355, 1275, 1200), 1050, 790
NMR ($CDCl_3$, TMS = 0.00 ppm) 2.3s 6H, 2.6t 2H, 2.9s 6H, 4.2t 2H.

Capillary gas chromatography–mass spectroscopy revealed a sample composition of approximately 95% purity that yielded a base peak of m/z 58, M + 1 fragment of m/z 161, and M − 1 fragment of m/z 159.

DMCC was prepared by adding 0.009 mol methyl iodide dropwise to a solution of 0.006 mol DMAE dimethylcarbamate in 50 mL of dry acetone. The slurry that formed was stirred for an additional 1.5 hr, then filtered and washed with 30 vol. of acetone followed by 30 vol. of diethyl ether, and dried. The yield was approximately 89%. The fluffy, white, crystalline material was soluble in ethanol, dimethyl sulfoxide (DMSO), and H_2O, and insoluble in acetone, diethyl ether and CH_2Cl_2. The product was used without further purification and stored in a dessicator at room temperature. Analytic: ($C_8H_{19}N_2O_2$)C,H,N,O.

Synthesis of DMAE carbamates. Various DMAE carbamates were prepared as described elsewhere [2]. Briefly, methyl, ethyl, propyl, butyl and phenyl isocyanates were refluxed for 6 hr in dry toluene with dimethylaminoethanol. The solvent and residual isocyanate were removed by evaporation *in vacuo*. The quaternary salts were prepared by reaction with methyl iodide.

Measurement of [^{3}H]nicotine, [^{3}H]methylcarbamylcholine ([^{3}H]MCC) and [^{3}H]3-quinuclidinyl benzilate ([^{3}H]QNB) binding. The procedure for the preparation and measurement of specific [^{3}H]nicotine, [^{3}H]MCC, and [^{3}H]QNB binding has been described [5]. Membranes are obtained from whole rat brain after homogenization in 0.05 M $NaPO_4$, pH 7.0, and centrifugation at 50,000 *g* for 30 min. The pellet is resuspended in phosphate buffer and stored