Nickel Carbonyl Inhibition of Induction of Aminopyrine Demethylase Activity in Liver and Lung

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SUMMARY

Exposure of rats to nickel carbonyl, Ni(CO)₄, in LD₅₀ dosage inhibited the basal (noninduced) levels of hepatic aminopyrine demethylase activity. The maximum inhibition of hepatic aminopyrine demethylase occurred on the 2nd day after an injection of Ni(CO)₄, and aminopyrine demethylase activity returned to normal levels by the 4th day after Ni(CO)₄. Administration of Ni(CO)₄ also inhibited hepatic aminopyrine demethylase activity in rats that had received daily injections of phenobarbital beginning 5 days before the Ni(CO)₄. However, in the phenobarbital-treated rats, maximum inhibition of hepatic aminopyrine demethylase did not occur until the 5th day after Ni(CO)₄ and aminopyrine demethylase activity did not return to normal levels until the 9th day after Ni(CO)₄. These experiments and other related studies indicate that Ni(CO)₄ inhibition of hepatic aminopyrine demethylase activity follows a distinctly different temporal sequence in noninduced and phenobarbital-treated rats.

The mean activity of aminopyrine demethylase activity in rat lungs was increased 2 days after ingestion of phenothiazine. Exposure of rats to Ni(CO)₄ inhibited the phenothiazine induction of pulmonary aminopyrine demethylase activity.

INTRODUCTION

Investigations in our laboratory have shown that administration of nickel carbonyl, Ni(CO)₄, to rats by inhalation or i.v. injection inhibits induction of liver enzymes (32—35). Three examples of this effect have been reported previously, with phenothiazine induction of benzpyrene hydroxylase (32), cortisone induction of tryptophan pyrrolase (33), and phenobarbital induction of cytochrome P-450 (34), as the experimental systems. In these studies, administration of Ni(CO)₄ in LD₅₀ dosage did not inhibit the basal (noninduced) activities of benzpyrene hydroxylase, tryptophan pyrrolase, or cytochrome P-450. Nickel carbonyl did block the pharmacological induction of each of these hepatic enzymes, with maximum inhibition occurring on the 1st or 2nd days after exposure to Ni(CO)₄ and gradual return of induced activities to normal by the 5th day (32—35). Nickel carbonyl did not affect substrate induction of tryptophan pyrrolase, which suggests that Ni(CO)₄ produces a metabolic block at the level of mRNA (33). This hypothesis has been supported by our observations that Ni(CO)₄ inhibits hepatic RNA polymerase activity (4, 36) and inhibits the incorporation of orotic acid-¹⁴C into hepatic RNA (3) but produces only slight diminution in the incorporation of leucine-¹⁴C into hepatic microsomal proteins (37).

The present study is primarily concerned with the effects of Ni(CO)₄ upon phenobarbital induction of hepatic aminopyrine demethylase activity, one of the drug-metabolizing functions of the microsomal oxidase system (20). Aminopyrine demethylase activity was selected for study since it requires integrated functioning of the entire sequence of enzymes which comprise the microsomal mixed-function oxidase system (7, 24). Moreover, there is an intimate interplay between phenobarbital induction of the microsomal oxidase system and phenobarbital stimulation of proliferation of smooth membranes of endoplasmic reticulum (1, 2, 5, 26, 27). As might be anticipated from the complexity of these related biochemical and ultrastructural processes, the effects of Ni(CO)₄ upon induction of hepatic aminopyrine demethylase activity have been found to be considerably more complicated than those that were previously observed in the other inducible enzyme systems (32—35).

MATERIALS AND METHODS

Measurements of aminopyrine demethylase activity in preparations from rat liver and lung were performed by an adaptation of the method of La Du et al. (20), incorporating modifications suggested by Fouts and Waters (8). Male rats of the Sprague-Dawley strain (90 to 100 g) were fasted for 17 hr before being killed by guillotine. Six to 10 treated rats were included in each experimental group, and 2 or more untreated control rats were tested in each day's experiment. The liver and lungs were excised, weighed, washed in 0.1 M Tris buffer (pH 7.5), and minced with scissors. The minced tissues from each rat were homogenized individually (10 or 20% homogenate, w/v, in 0.1 M Tris buffer, pH 7.5) for 15 to 20 sec in a motor-driven Teflon-glass tissue grinder. The homogenates were centrifuged (20 min, 9000 X g), and the
supernatant fractions were decanted and immediately assayed for aminopyrine demethylase activity. The guillotine, dissection, homogenization, and centrifugation steps were performed in a cold room at 4–10°C.

For each assay, 25-ml Erlenmeyer flasks were prepared in triplicate containing: (a) 0.5 M Tris buffer, pH 7.5 (1 ml); (b) 1 M nicotinamide (0.1 ml); (c) 0.5 M MgSO₄ (0.15 ml); (d) 2 mM TPN (0.1 ml); (e) 0.25 M glucose 6-phosphate (0.1 ml); (f) 10 mM aminopyrine (1 ml); (g) liver 9000 X g supernatant fraction (0.2 to 1 ml) or lung 9000 X g supernatant fraction (1 to 2 ml); and (h) H₂O to a final volume of 5 ml. Trichloroacetic acid, 30% w/v (1 ml), was added to 1 flask ("enzyme blank" sample), and the flask was placed in an ice bath. The remaining 2 flasks ("enzyme reaction" samples) were shaken vigorously (100 oscillations/min) for 60 min in a shaking water bath at 37°C. The enzymatic reaction was stopped by addition of trichloroacetic acid, 30%, w/v (1 ml). The contents of all of the flasks were transferred to centrifuge tubes, and the precipitated proteins were sedimented by centrifugation (15 min, 1200 X g). Four ml of each protein-free supernatant were transferred to glass-stoppered centrifuge tubes that contained 1 ml trichloroacetic acid, 6% w/v. Reagent blank and standard samples were prepared in duplicate in similar centrifuge tubes. The reagent blanks consisted of 5 ml trichloroacetic acid, 6%, w/v. The standard samples consisted of 5 μg 4-aminoantipyrine in 4 ml water, plus 1 ml trichloroacetic acid, 30%, w/v. Sodium nitrite, 0.2%, w/v (0.5 ml), was added to all of the centrifuge tubes. After 10 min in an ice bath, ammonium sulfamate solution, 1%, w/v (0.5 ml), was added. After 3 min at room temperature, 1-naphthol solution, 5%, w/v (0.1 ml), and 4 N NaOH (1 ml) were added. After 10 min at room temperature, concentrated HCl reagent (0.5 ml) and isoamyl alcohol (2 ml) were added. The tubes were stoppered, mixed with a Vortex rotary mixer for 1 min, and centrifuged (10 min, 1200 X g). The supernatant organic phases were transferred to spectrophotometer cuvets, and the absorbances were measured at 540 μm. Under the reaction conditions, the calibration curve was linear throughout the range of measurements, and enzymatic activity was constant throughout the period of incubation. The absorbance which was obtained with the "enzyme blank" sample was subtracted from the mean absorbance of the samples which had been incubated for 60 min. Aminopyrine demethylase activity was expressed in units equivalent to the formation of 1 μg 4-aminoantipyrine/g wet tissue/hr.

Administration of Ni(CO)₄ by inhalation and by i.v. injection was performed as previously described (11, 12). The LC₅₀ exposure to Ni(CO)₄ by inhalation was 0.20 mg Ni/liter air/15 min. The LD₅₀ dosage by injection into a tail vein with a microsyringe was 2.2 mg Ni/100 g body weight (equivalent to 5 μl Ni(CO)₄/100 g). LD₅₀ or LC₅₀ exposures to Ni(CO)₄ were used throughout the present study, except for 1 experiment in which a dose-response curve was obtained. Phenobarbital was injected i.p. (7.5 mg/100 g), according to daily dosage schedules previously used for induction of hepatic cytochrome P-450 (34). Cortisone acetate was injected i.p. (10 mg/100 g) at 3 and 6 hr before sacrifice, as previously done for induction of hepatic tryptophan pyrrolase (33). Phenothiazine was administered by gastric intubation (10 mg/100 g) 48 hr before sacrifice, as previously done for induction of pulmonary and hepatic benzpyrene hydroxylase (32).

RESULTS

Aminopyrine demethylase activity in livers of untreated control rats averaged 56 units (S.D. = ±8; N = 23). Four hr after the initial injection of phenobarbital, there was no significant increase in aminopyrine demethylase activity (mean = 62 units, S.D. = ±10; N = 6). Twenty-four hr after injection of phenobarbital, aminopyrine demethylase activity averaged 97 units (S.D. = ±5; N = 6; p < 0.01). Induction of aminopyrine demethylase activity was almost complete by 48 hr, (mean = 134 units; S.D. = ±8; N = 6; p < 0.01), and reached maximum levels (mean = 152 units; S.D. = ±13; N = 6; p < 0.01) on the 4th day. The induced levels of aminopyrine demethylase activity were maintained at a plateau of 150 units (S.D. = ±10; N = 28; p < 0.01), for as long as 3 weeks of daily phenobarbital administration.

The effects of i.v. injection of Ni(CO)₄ upon hepatic aminopyrine demethylase activities are shown in Chart 1.
aminopyrine demethylase activity (mean = 52 units; S.D. = ±3; N = 7). At 24 hr, there was a diminution in enzyme activity, which progressed to a minimum of 6.5 units (S.D. = ±4.3; N = 10) on the 2nd day after injection of Ni(CO)₄. The enzyme activity returned to the normal range by the 4th day.

A different pattern of inhibition was observed in the phenobarbital-treated rats. At 4 hr after the injection of Ni(CO)₄, there was no significant inhibition of hepatic aminopyrine demethylase activity (mean = 153; S.D. = ±17; N = 6). The activity began to diminish on the 1st and 2nd days after Ni(CO)₄, paralleling the diminution which was found in noninduced rats. However, instead of returning to normal, there was abrupt diminution of aminopyrine demethylase activity on the 4th and 5th days to a level (mean on 5th day = 16 units; S.D. = ±3; N = 5), which was significantly less than the corresponding level in noninduced rats (p < 0.01). Beginning on the 6th day, there was gradual return of enzyme activity to the normal induced range by the 10th day after injection of Ni(CO)₄.

After exposure to Ni(CO)₄ by inhalation, the patterns of inhibition of hepatic aminopyrine demethylase activities in noninduced and in chronically induced rats closely resembled the patterns which were produced by i.v. injection of Ni(CO)₄. In the noninduced rats, the minimum hepatic aminopyrine demethylase activity occurred on the 2nd day after inhalation (mean = 15 units; S.D. = ±3; N = 4), and the enzyme activity returned to the normal range by the 4th day. In the phenobarbital-treated rats, the minimum hepatic aminopyrine demethylase activity occurred on the 5th day after inhalation (mean = 6 units; S.D. = ±4; N = 5), and the enzyme activity gradually returned to the normal induced range by the 10th day after inhalation of Ni(CO)₄.

An experiment which measured the effect of Ni(CO)₄ upon the capacity for phenobarbital induction of hepatic aminopyrine demethylase activity is illustrated in Chart 2. Sixty rats were given Ni(CO)₄ by i.v. injection. Thirty of these rats, evenly distributed into 6 groups, were killed as follows: (a) immediately; (b) 1 day; (c) 2 days; (d) 3 days; (e) 4 days; and (f) 5 days after injection of Ni(CO)₄. The remaining 30 rats were distributed into 6 additional groups which were killed on the 2nd, 3rd, 4th, 5th, 6th, and 7th days after the injection of Ni(CO)₄. These latter groups of rats were given phenobarbital injections on the 2 days before sacrifice. Therefore, the dashed lines and arrowheads in Chart 2 indicate the capacity for phenobarbital induction of hepatic aminopyrine demethylase activity on successive days after the injection of Ni(CO)₄. Administration of phenobarbital, beginning concurrently with the Ni(CO)₄ injection, or on the 1st day thereafter, produced normal induction of aminopyrine demethylase activity. In contrast, essentially no induction was found when the phenobarbital administration was begun on the 2nd, 3rd, or 4th days after Ni(CO)₄, despite the fact that the basal level of aminopyrine demethylase activity had returned to normal by the 4th day. The capacity for phenobarbital induction of hepatic aminopyrine demethylase began to reappear on the 5th day after the injection of Ni(CO)₄.

A dose-response curve for the inhibitory effect of Ni(CO)₄ upon phenobarbital induction of hepatic aminopyrine demethylase is illustrated in Chart 3. Nickel carbonyl was administered to 5 groups of 4 rats in i.v. dosages of 0.4, 0.8, 1.2, 1.6, and 2.0 mg Ni/100 g body weight on the 4th day before sacrifice. A control group received sham injections. Phenobarbital was administered to all the rats on the 2 days before sacrifice. The mean activity of aminopyrine demethylase in the livers of the control rats was 136 units (S.D. = ±4). At the lowest dosage level (0.4 mg Ni/100 g), there was 59% diminution of enzyme activity (mean = 56 units; S.D. = ±8). At the approximate LD₅₀ dosage level (2.0 mg Ni/100 g), there was 86% diminution of enzyme activity (mean = 19 units; S.D. = ±3).
As a test for the possibility that anorexia could cause deficiency of a nutrient which might be required for aminopyrine demethylase activity, 4 rats were maintained for 48 hr on a diet consisting solely of 5% glucose solution, w/v, ad libitum. The aminopyrine demethylase activity in livers from these rats averaged 53 units (S.D. = ±3), which did not differ significantly from the activity in normal controls, (mean = 56 units; S.D. = ±8).

An attempt was made to demonstrate an inhibitor of aminopyrine demethylation in liver homogenates of rats that had received Ni(CO)₄. Rat A received phenobarbital on the 2 days before sacrifice; its hepatic aminopyrine demethylase activity was 124 units. Rats B and C received Ni(CO)₄ i.v. 4 days before sacrifice and phenobarbital on the 2 days before sacrifice. Hepatic aminopyrine demethylase activities in these rats were 10.2 and 12.5 units, respectively. The activities observed when portions of liver Homogenate A were mixed with equal volumes of Homogenates B or C were 67.5 and 69.4 units, respectively, which corresponded closely to the calculated values of 67.1 and 68.3 units which were obtained by adding activities A + B or A + C, and dividing by 2. Thus, this experiment failed to reveal any inhibitor of aminopyrine demethylation in liver homogenates of Ni(CO)₄-treated rats. Nickel chloride was added in vitro in concentrations from 10⁻³ to 10⁻⁹ M to enzyme reaction mixtures containing 9000 X g liver supernatant fraction of homogenate A from groups which had received phenobarbital injections for 5 days. In both the noninduced and phenobarbital-treated rat livers, there was 10% inhibition of aminopyrine demethylase activity at a Ni²⁺ concentration of 10⁻⁷ M, 25% inhibition at 10⁻⁸ M, and 50% inhibition at 10⁻⁹ M. [For comparison, the mean Ni²⁺ concentration in enzyme reaction mixtures containing 9000 X g supernatant fraction from rats sacrificed 24 hr after i.v. Ni(CO)₄ was 5 X 10⁻⁷ M, computed from measurements of ⁶³Ni in subcellular fractions of rat liver following i.v. injections of ⁶³Ni(CO)₄ (17, 39).] The inhibitory effect of Ni²⁺ upon aminopyrine demethylase activity in vitro was of the noncompetitive type, as determined from Lineweaver-Burk plots (6) of the reciprocals of reaction velocities and substrate concentrations in the presence of Ni²⁺ concentrations ranging from 10⁻³ to 10⁻⁷ M. The Kᵣ value for aminopyrine (6.4 X 10⁻⁸ M) was not significantly altered by the in vitro additions of Ni²⁺, nor by in vivo injections of phenobarbital or Ni(CO)₄, singly or in combination, in any of the experimental groups.

As shown in Chart 4, no induction of hepatic aminopyrine demethylase activity was observed after administration of cortisone acetate or phenothiazine in the dosages which were previously used for induction of hepatic tryptophan pyrrolase (33) and benzpyrene hydroxylase (32). There was no detectable aminopyrine demethylase activity in lungs from control rats. Aminopyrine demethylase activity in the lung was induced by phenothiazine, but not by phenobarbital or cortisone acetate. Forty-eight hr after ingestion of phenothiazine, the mean activity of pulmonary aminopyrine demethylase was 6.7 units (S.D. = ±1.3; N = 14).

The finding that phenothiazine stimulated pulmonary aminopyrine demethylase activity provided an experimental method for the study of the effect of Ni(CO)₄ upon enzyme induction in the lung, (Chart 5). Pulmonary aminopyrine demethylase activity was somewhat diminished in rats sacrificed 2 days after i.v. Ni(CO)₄ i.e., rats in which Ni(CO)₄ and phenothiazine were administered simultaneously. In the lungs of rats that were sacrificed from 3 to 7 days after Ni(CO)₄, no aminopyrine demethylase activity was detected. Induced aminopyrine demethylase activity reappeared in the lung on the 8th day after Ni(CO)₄.

**DISCUSSION**

During the months since these experiments were completed, Ichii and Yago (15), and Orenius et al. (25) have independently discovered that corticosteroids and androgens are necessary to maintain basal (noninduced) levels of aminopyrine demethylase activity in livers of male rats. Following adrenalectomy and/or castration of male rats, there is prompt diminution of hepatic aminopyrine demethylase activity (15, 25). The diminution of aminopyrine demethylase activity is prevented or reversed by hormonal replacement therapy. Furthermore, Ichii and Yago (15) have demonstrated that the phenobarbital-inducible
fraction of hepatic aminopyrine demethylase activity is completely independent of adrenal and testicular control. Since adrenal insufficiency has been observed in rats following exposures to Ni(CO)₄ (31), we suspect that the diminution of hepatic aminopyrine demethylase activity in noninduced rats on the 1st to 3rd days after Ni(CO)₄ may represent a secondary effect of acute adrenocortical (and possibly testicular) insufficiency. This could also account for the mild diminution of hepatic aminopyrine demethylase activity which was seen in chronically induced rats on the 1st to 3rd days after Ni(CO)₄. In the latter case, it would be assumed that only the hormone-dependent (noninduced) component of total aminopyrine demethylase activity was diminished, and that the phenobarbital-induced component remained essentially unimpaired until the 4th day after Ni(CO)₄. To test these speculations, we plan to investigate phenobarbital-treated rats after exposure to Ni(CO)₄.

As an alternative hypothesis, the diminished enzyme activity which occurred in noninduced rats on the 1st to 3rd days after Ni(CO)₄ may be caused by severe dilation of RER. Hackett and Sunderman (13) have found that dilation of RER is the most striking cytoplasmic alteration which develops in rat hepatocytes after injection of Ni(CO)₄. There appears to be a close temporal association between the distention of RER and the inhibition of hepatic aminopyrine demethylase activity in noninduced rats. In hepatocytes of noninduced rats, aminopyrine demethylase activity is present in both RER and SER (28), but the rough membranes are far more abundant than smooth membranes (26). Since aminopyrine demethylase activity involves an integrated chain of enzymes which are believed to be clustered at adjacent loci on membranes of the endoplasmic reticulum (19, 27), it can readily be visualized that the dilation of RER which is produced by Ni(CO)₄ might temporarily disrupt the coupled enzyme reactions. In this situation, aminopyrine demethylase activity could spontaneously reappear when the distention of RER begins to diminish on the 4th day after Ni(CO)₄ (13).

Gram et al. (10) have shown that the increased aminopyrine demethylase activity which occurs after administration of phenobarbital is located entirely in SER. No ultrastructural lesions were found in SER after exposure to Ni(CO)₄ (13). Therefore, the mild diminution of hepatic aminopyrine demethylase activity which occurred in chronically induced rats on the 1st or 3rd days after Ni(CO)₄ may represent selective inhibition of the noninduced enzyme activity in dilated RER, while the induced enzyme activity in SER remained essentially unaffected.

As shown in Chart 2, the inhibitory effect of Ni(CO)₄ upon 48-hr phenobarbital induction of hepatic aminopyrine demethylase activity was first detected in rats that were sacrificed 4 days after the injection of Ni(CO)₄. Thus, the capacity for phenobarbital induction was unimpaired for at least 24 hr after Ni(CO)₄. Our previous studies have shown that RNA polymerase activity in hepatic nuclei is inhibited at least 6 hr after Ni(CO)₄ (4) and that the inhibition persists for at least 24 hr (35). The initiation of phenobarbital induction of aminopyrine demethylase activity was therefore unimpaired at a time when RNA synthesis was profoundly depressed. In addition to stimulating RNA polymerase activity in hepatic nuclei (9, 36), phenobarbital has been reported to increase the rate of translation of synthetic messenger RNA by hepatic microsomes (18), and to decrease the rates of catabolism of microsomal RNA (21), phospholipids (14), enzymes (16, 22, 29) and other proteins (19, 30). These cytoplasmic effects of phenobarbital apparently can permit induction of aminopyrine demethylase activity to continue after an exposure to Ni(CO)₄, until the time when deficiency of mRNA for any of the coupled microsomal enzymes becomes rate limiting. We believe that the unresponsiveness of hepatic aminopyrine demethylase activity to phenobarbital stimulation from the 2nd to the 4th days after Ni(CO)₄ was a manifestation of depletion of mRNA templates, owing to Ni(CO)₄ inhibition of RNA polymerase activity (3, 4, 36).

As shown in Chart 1, on the 4th and 5th days after Ni(CO)₄, hepatic aminopyrine demethylase activity in chronically-induced rats fell significantly below the corresponding levels in noninduced rats. Therefore, in addition to a delayed inhibitory effect of Ni(CO)₄ upon phenobarbital induction of hepatic aminopyrine demethylase activity, there appears to be an inhibitory effect of phenobarbital upon recovery from the hormonal insufficiency or upon repair of the ultrastructural lesion of RER produced by Ni(CO)₄. These possibilities can be tested experimentally and will be investigated in future studies in our laboratory.

Our observation that administration of phenothiazine causes induction of pulmonary aminopyrine demethylase activity has received independent support by subsequent studies of Oppelt et al. (23). These investigators have found that chlorpromazine administration caused 30 to 50% increase in aminopyrine demethylase activity in rabbit lung, whereas phenobarbital administration had only a very small stimulatory effect upon the activity of pulmonary aminopyrine demethylase. Our studies, as well as those of Oppelt et al. (23), suggest that the lung may play a role in the enzymatic metabolism of drugs. Moreover, it appears that different pharmacological agents are responsible for induction of the microsomal oxidase systems in lung and in liver.

REFERENCES


2The abbreviations used are: RER, rough endoplasmic reticulum; SER, smooth endoplasmic reticulum.
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