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FURTHER EVIDENCE OF CYCLIC AMP-MEDIATED HYPERTROPHY AS A PREREQUISITE OF DRUG-SPECIFIC ENZYME INDUCTION

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Abstract—We have previously reported that the administration of phenobarbital, 3-methylcholanthrene or the polychlorinated biphenyl, Aroclor-1254, to rats resulted in an early and sequential increase in the activities of hepatic cAMP-dependent protein kinase(s), ornithine decarboxylase and RNA polymerase I. It was suggested that this sequence of events was involved in both liver hypertrophy and the induction of microsomal mixed-function oxygenases. To further test this hypothesis, we investigated if mice unable to induce aryl hydrocarbon hydroxylase in response to 3-methylcholanthrene exhibited increases in these cAMP-mediated events. A single dose of 3-methylcholanthrene (150 mg/kg, i.p.) was administered to male C57Bl/6J (aryl hydrocarbon responsive) and DBA/2J (aryl hydrocarbon nonresponsive) mice. In the C57Bl/6J mice, the hepatic cAMP concentration increased to 165 per cent of control within 1 hr. Maximal increases in the activities of liver cAMP-dependent protein kinase(s) (160 per cent), ornithine decarboxylase (210 per cent) and RNA polymerase I (120 per cent) occurred at 2, 4 and 6 hr, respectively, in the responsive mice. There were no detectable increases in any of these parameters in the nonresponsive (DBA/2J) mice. Multiple doses of 3-methylcholanthrene (20 mg/kg, i.p., daily × 3) resulted in increases in hepatic aryl hydrocarbon hydroxylase (460 per cent) and liver weight/body weight ratios (118 per cent) in the responsive (C57Bl/6J) mice killed at 5 days. There was no increase in either of these parameters in the nonresponsive (DBA/2J) mice. Both the responsive and nonresponsive mice responded similarly to a single parental dose of phenobarbital (100 mg/kg) with maximal increases in the activities of cAMP-dependent protein kinase(s) (150 per cent), ornithine decarboxylase (160 per cent) and RNA polymerase I (135 per cent) at 2, 4 and 8 hr respectively. Multiple doses of phenobarbital (100 mg/kg, i.p., daily × 3) resulted in increased ethylmorphine *N*-demethylase activity (160 per cent) and liver weight/body weight ratios (130 per cent) in both strains of mice at 5 days. These data provide further evidence of coupled cAMP-mediated hypertrophy and induction of mixed-function oxygenases in liver.

Aryl hydrocarbon [benzo(a)pyrene] hydroxylase is a microsomal mono-oxygenase which metabolizes polycyclic hydrocarbons, as well as other xenobiotics [1–5]. Enzyme activity can be induced in the liver of certain inbred strains of mice termed aryl hydrocarbon responsive by the administration of polycyclic hydrocarbons. In other inbred strains of mice, termed aryl hydrocarbon nonresponsive, hepatic aryl hydrocarbon hydroxylase cannot be induced by the administration of these compounds [6, 7].

The ability to induce aryl hydrocarbon hydroxylase in response to the administration of polycyclic hydrocarbons segregates as a Mendellian autosomal dominant gene, which appears to code for a cytosol protein, the "induction receptor" [7, 8]. It has been postulated that the binding of the inducing compound to the receptor protein initiates a series of events that results in de novo synthesis of aryl hydrocarbon hydroxylase [8]. As the result of a mutation, aryl hydrocarbon nonresponsive mice have an altered receptor protein with diminished affinity for inducing compounds [8]. These mice, therefore, are an excellent control for the delineation of the biochemical alterations involved in the induction of these hepatic microsomal enzymes.

The induction of drug-metabolizing enzymes by the administration of xenobiotics has been shown to occur in conjunction with the general process of liver hypertrophy [9–11]. The molecular mechanisms by which xenobiotics induce liver growth and the production of microsomal drug-metabolizing enzymes are unknown, although many specific enzyme changes have been noted [9–12]. We have recently proposed a model suggesting that both the induction of microsomal mixed-function oxygenases and hypertrophy are the result of a general series of biochemical events initiated by an increase in the intracellular concentration of cAMP and/or the activation of cAMP-dependent protein kinase [14] (Fig. 1). Since cAMP is rapidly synthesized and rapidly degraded, determining the degree of activation of cAMP-dependent protein kinase (ATP-protein phosphotransferase, EC 2.7.1.37), the means by which cAMP exerts intracellular control [15, 16], is the most reliable measure of a cAMP-mediated event. The increased activation of cAMP-dependent protein kinase in response to trophic stimuli is consistently followed by an elevation in the activity of ornithine decarboxylase (L-ornithine carboxy-lase, EC 4.1.1.17). There is now substantial evidence that ornithine decarboxylase is induced in

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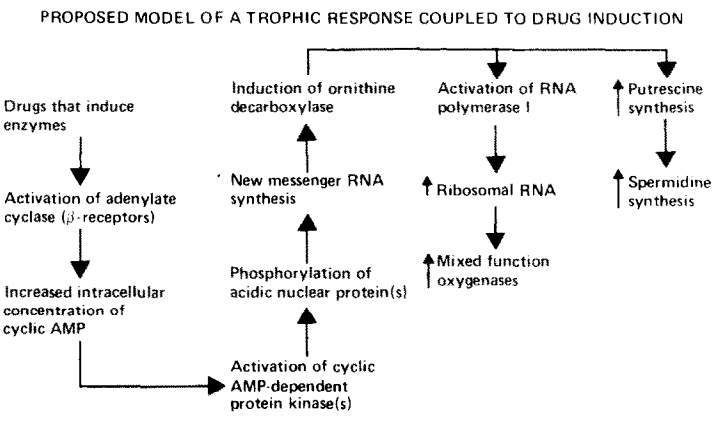

Fig. 1. Proposed model of the major events which couple liver hypertrophy and specific enzyme induction after drug administration.

response to the activation of cAMP-dependent protein kinase [14, 17, 18]. Ornithine decarboxylase, in turn, has recently been suggested as the labile protein that allows RNA polymerase I (a nucleoside triphosphate-RNA nucleotidyl transferase, EC 2.7.7.6) to initiate transcription [19–21]. Increased ornithine decarboxylase activity in stimulated tissues is rapidly followed by increased RNA polymerase I activity. These sequential events have been observed in every system examined to date, including several that result in the induction of microsomal mixed-function oxygenases and hypertrophy (e.g. in rat liver after the administration of phenobarbital [10, 12], polychlorinated biphenyls (Aroclor-1254) [11] and 3-methylcholanthrene [10, 12].

Since an activation of cAMP-dependent protein kinase appears to mediate or facilitate microsomal enzyme induction, we postulated that 3-methylcholanthrene would activate this kinase only in the aryl hydrocarbon responsive strain of mice. Similarly, the subsequent induction of ornithine decarboxylase and activation of RNA polymerase I would only occur in the responsive mice. In this manuscript, we report that administration of 3-methylcholanthrene to C57Bl/6J mice (aryl hydrocarbon responsive) does result in an elevation of hepatic cAMP followed sequentially by an activation of cAMP-dependent protein kinase, induction of ornithine decarboxylase and an activation of RNA polymerase I. None of these results were observed after administration of 3-methylcholanthrene to DBA/2J mice (aryl hydrocarbon nonresponsive). Both strains of mice have similar responses to phenobarbital, a drug which induces through the cytochrome P-450 pathway rather than the P-448 aryl hydrocarbon hydroxylase pathway of 3-methylcholanthrene [2].

MATERIALS AND METHODS

Animals. Mature male mice (20–25 g) of both the aryl hydrocarbon responsive (C57Bl/6J) and aryl hydrocarbon nonresponsive (DBA/2J) strains were purchased from Jackson Laboratories, Bar Harbor, ME. The mice were maintained on a 12-hr light–dark cycle and fed *ad lib.* Animals were injected i.p. with either phenobarbital (100 mg/kg in 0.9% saline) or 3-methylcholanthrene (150 mg/kg in corn oil). Controls received an equivalent amount (approximately 0.2 ml) of the appropriate vehicle. The animals were sacrificed between 10:00 and 11:00 a.m. to minimize diurnal variations in enzyme activities. Determinations of cAMP concentration, cAMP-dependent protein kinase activation, ornithine decarboxylase activity, and RNA polymerase I activity were all performed on samples from the same liver. The extent of hypertrophy, as well as determinations of the particular microsomal mixed-function oxygenase, was assessed after administering either phenobarbital (100 mg/kg, i.p., in 0.9% saline) or 3-methylcholanthrene (150 mg/kg, i.p., in corn oil) for 3 consecutive days and then comparing liver weight/body weight ratios on day 5. Measurements of the microsomal mixed-function oxygenases were performed on liver samples from these mice.

Cyclic AMP determinations. Cyclic AMP levels were determined in liver samples dropped into liquid nitrogen within 5 sec of killing the animals. Cyclic AMP was extracted into 0.4 N perchloric acid, purified by aluminum oxide and Dowex chromatography according to Mao and Guidotti [22] and assayed by measuring its ability to activate purified beef heart protein kinase [23].

Cyclic AMP-dependent protein kinase activity ratio. Liver samples (approximately 0.2 g) were frozen within 10 sec of sacrifice. The samples were homogenized in 4 vol. of 0.01 M phosphate buffer, pH 6.5, containing 5 mM EDTA, 1 mM aminophylline, and 150 mM NaCl and centrifuged at 50,000 *g* for 15 min. The resulting supernatant was diluted 1:5 with the homogenizing buffer and 25 μl used as the enzyme source for the protein kinase assay. The assay mixture contained 25 mM phosphate buffer, pH 6.5, 5 mM NaF, 15 mM Mg acetate, 3 mg/ml of calf thymus histone mixture, 0.1 mM [γ-^{32}P]ATP (4.8 Ci/m-mole, New England Nuclear, Boston, MA), 1 mM aminophylline, and when added, 5 μM cAMP, in a final volume of 100 μl. The reaction was initiated by the addition of the enzyme preparation, allowed to incubate for 10 min at 30° and terminated by pipeting 50-μl aliquots of the reaction mixture onto Whatman

2.3 cm 3 MM filter paper discs. The discs were dropped into a stirring 10% trichloroacetic acid bath and washed as described by Reiman *et al.* [24].

The activity of protein kinase is expressed as the ratio of activity in the absence and presence of exogenous saturating cAMP (− cAMP/+ cAMP). This ratio reflects the intracellular degree of activation of the cAMP-dependent protein kinase [25].

Ornithine decarboxylase activity. Ornithine decarboxylase activity was determined by measuring the liberation of $^{14}CO_2$ from D,L,-[1-^{14}C]ornithine (5.3 mCi/m-mole, New England Nuclear, Boston, MA [26]. The buffer used in the assay was 50 mM sodium-potassium phosphate, pH 7.2, containing 0.1 mM EDTA and 1.0 mM dithiothreitol.

RNA polymerase I activity. Using a purified nuclear preparation as the enzyme source [27], RNA polymerase activity was determined by measuring the incorporation of [^{3}H]UTP (18.6 Ci/m-mole, Schwartz/Mann, Orangeburg, NY) into RNA [28]. RNA polymerase I activity was separated from RNA polymerase II activity by assaying in the presence and absence of 1.8 μg/ml of α-amanitin [29].

Microsomal enzymes. Ethylmorphine *N*-demethylase activity was measured as previously described [30]. Formaldehyde was measured by the method of Nash [31]. Benzo(a)pyrene hydroxylase activity was determined in the 9000 *g* supernatant as described by Nebert and Gelboin [32]. The concentration of the hydroxylated benzo(a)pyrene was assayed spectrophotofluorometrically using 3-hydroxy-benzo(a)pyrene as the standard (generously supplied by H. V. Gelboin of N.I.H. and Wayne Levin of Hoffmann-LaRoche, Inc.).

Protein determinations. Microsomal protein concentrations were determined using the Biuret procedure [33]. All other protein concentrations were measured according to the method of Lowry *et al.* [34] using bovine serum albumin as the standard.

RESULTS

Administration of 3-methylcholanthrene to C57Bl/6J and DBA/2J mice. Within 1 hr after the administration of 3-methylcholanthrene to C57Bl/6J mice, the hepatic cAMP level increased from 0.40 to 0.67 pmole/mg of liver (Fig. 2). The cAMP-dependent protein kinase activity ratio in this same strain increased from 0.2 to 0.35 within 2 hr of drug administration (Fig. 3). In contrast, there was no similar increase in hepatic cAMP concentration nor any change in the activity ratio of cAMP-dependent protein kinase in DBA/2J mice. Increases in ornithine decarboxylase activity and RNA polymerase I activity were detectable only in the C57Bl/6J mice after 3-methylcholanthrene administration. Ornithine decarboxylase activity was significantly elevated within 2 hr in the C57Bl/6J mice, was elevated over 2-fold within 4 hr, and then declined toward the control value (Fig. 4). Compared to the nonresponsive strain, RNA polymerase I was significantly increased within 4 hr of drug administration and remained elevated through 8 hr, the last time that it was measured (Fig. 4).

In order to assess liver hypertrophy as well as the extent of the induction of mixed-function oxygenases,

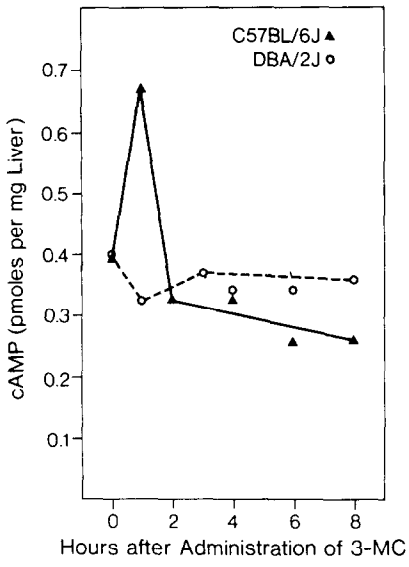

Fig. 2. Comparison of the changes in hepatic cAMP concentration after a single dose of 3-methylcholanthrene in both C57Bl/6J and DBA/2J mice. Male mice (20–25 g) received 3-methylcholanthrene (3-MC) (150 mg/kg in corn oil, i.p.). The animals were sacrificed by cervical dislocation at the indicated intervals and the concentration of cAMP in each liver was determined [22, 23]. Each point represents the mean for determinations on three animals.

the two strains of mice received daily injections of 3-methylcholanthrene (20 mg/kg, i.p.) for 3 days. The mice were killed on day 5, and hepatic benzo(a)pyrene hydroxylase activity was measured, as well as assess-

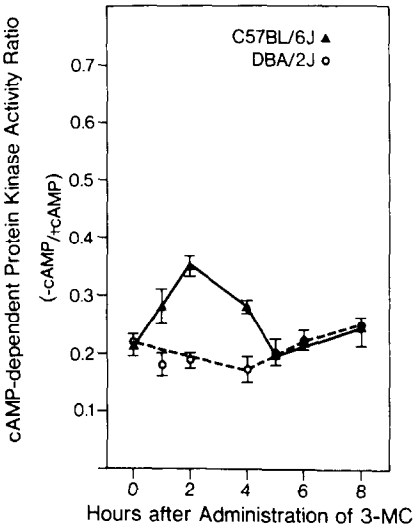

Fig. 3. Comparison of the changes in hepatic cAMP-dependent protein kinase activity ratio after a single dose of 3-methylcholanthrene in both C57Bl/6J and DBA/2J mice. Male mice (20–25 g) received 3-methylcholanthrene (3-MC) (150 mg/kg in corn oil, i.p.). The animals were sacrificed at the indicated intervals and each liver was assayed for enzyme activity by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into histones in the absence and presence of exogenous cAMP (−cAMP/+cAMP) [24, 25]. Each point represents the mean ± S. E. M. for duplicate determinations on three animals.

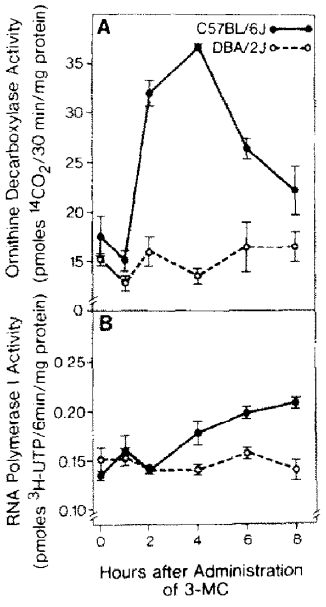

Fig. 4. Comparison of the changes in hepatic ornithine decarboxylase activity (A) and RNA polymerase I activity (B) after the administration of 3-methylcholanthrene in both C57Bl/6J and DBA/2J mice. Male mice (20–25 g) received 3-methylcholanthrene (3-MC) (150 mg/kg in corn oil, i.p.). Animals were sacrificed at the indicated intervals by cervical dislocation and each liver was assayed for ornithine decarboxylase and RNA polymerase I activities. Ornithine decarboxylase activity was determined by measuring the liberation of $^{14}CO_2$ from D,L-[1-^{14}C]ornithine [26]. RNA polymerase I was assayed in purified nuclear preparations by measuring the incorporation of [^{3}H]UTP into RNA in the presence of α-amanitin [27–29]. Each point represents the mean ± S. E. M. for triplicate determinations on three animals.

ing the liver weight/body weight ratio. There was over a 5-fold increase in benzo(a)pyrene hydroxylase activity in the aryl hydrocarbon responsive mice (Table 1) accompanied by a significant increase in the liver weight/body weight ratio (Table 2). No significant change in either of these parameters was detectable in the aryl hydrocarbon nonresponsive mice.

Table 1. Changes in hepatic benzo(a)pyrene hydroxylase activity in C57Bl/6J and DBA/2J mice after treatment with 3-methylcholanthrene*

Strain	Benzo(a)pyrene hydroxylase activity (nmoles/mg microsomal protein/10 min)	
	Control	3-Methylcholanthrene
C57Bl/6J	4.3 ± 0.33	19.8 ± 0.8†
DBA/2J	3.4 ± 0.12	5.0 ± 0.2†

* Male mice of the C57Bl/6J and DBA/2J strains received 3-methylcholanthrene (20 mg/kg, i.p., in corn oil) daily × 3. Controls received the injection vehicle alone. On day 5, all animals were sacrificed and the livers removed and asssyed for enzyme activity. The values are the means ± S. E. M. for six animals.

† Data differ from control ($P < 0.001$).

Administration of phenobarbital to C57Bl/6J and DBA/2J mice. Table 3 summarizes the changes in the cAMP-dependent protein kinase activity ratio, ornithine decarboxylase activity, and RNA polymerase I activity in the livers of aryl hydrocarbon hydroxylase responsive and aryl hydrocarbon hydroxylase nonresponsive mice after a single injection of phenobarbital. The cAMP-dependent protein kinase activity ratio was significantly elevated at 2 hr in both strains of mice, ornithine decarboxylase activity was significantly elevated by 4 hr in both strains, and significant activation of RNA polymerase I was detected in both strains by 6–8 hr.

The ratio of liver weight to body weight in the aryl hydrocarbon hydroxylase responsive and aryl hydrocarbon hydroxylase nonresponsive strain after phenobarbital administration was similar. That is, there was a significant increase in this ratio in both strains (Table 2). Further, after three injections of phenobarbital, ethylmorphine *N*-demethylase activity was significantly elevated in both strains (Table 4).

DISCUSSION

We have previously implicated a cAMP-mediated mechanism in the induction of hepatic drug-metabolizing enzymes [10–12, 14]. The administration of

Table 2. Changes in the ratio of liver weight/body weight in the C57Bl/6J and DBA/2J strains of mice after the administration of phenobarbital or 3-methylcholanthrene*

Drug	Strain	Control (g liver wt/ g body wt)	Experimental (g liver wt/ g body wt)
3-Methyl-cholanthrene	C57Bl/6J	5.45 ± 0.12	6.41 ± 0.13†
	DBA/2J	4.98 ± 0.09	4.86 ± 0.07
Phenobarbital	C57Bl/6J	5.47 ± 0.05	7.14 ± 0.45‡
	DBA/2J	5.06 ± 0.07	6.53 ± 0.17†

* Male mice (20–25 g) of the C57Bl/6J and DBA/2J strains received a series of three doses of either phenobarbital (100 mg/kg in 0.9% saline, i.p.) or 3-methylcholanthrene (20 mg/kg in corn oil, i.p.) at daily intervals. Controls received the appropriate solvent. Animals were weighed and sacrificed on day 5. The livers were removed, blotted dry and weighed. Each point represents the mean ± S. E. M. for seven or more animals.

† Data differ from control ($P < 0.001$).

‡ Data differ from control ($P < 0.005$).

Table 3. Changes in the cyclic AMP-dependent protein kinase activity ratio, ornithine decarboxylase activity and RNA polymerase I activity in the livers of C57Bl/6J and DBA/2J mice after phenobarbital*

Hr	cAMP-dependent protein kinase activity ratio (−cAMP/+cAMP)		Ornithine decarboxylase activity (pmoles $^{14}CO_2$/30 min/mg)		RNA polymerase I activity (pmoles [^{3}H]UTP/6 min/mg)	
	C57Bl/6J	DBA/2J	C57Bl/6J	DBA/2J	C57Bl/6J	DBA/2J
0	0.20 ± 0.03	0.20 ± 0.02	14 ± 3	15 ± 3	0.14 ± 0.10	0.13 ± 0.12
1	0.19 ± 0.02	0.20 ± 0.01	14 ± 2	18 ± 4	0.11 ± 0.08	0.13 ± 0.05
2	0.30 ± 0.02†	0.26 ± 0.01†	19 ± 3	19 ± 6	0.13 ± 0.06	0.14 ± 0.10
4	0.29 ± 0.03	0.29 ± 0.02	23 ± 2†	22 ± 1†	0.15 ± 0.08	0.13 ± 0.09
6	0.29 ± 0.03	0.26 ± 0.02	18 ± 4	18 ± 2	0.15 ± 0.10	0.12 ± 0.07‡
8	0.25 ± 0.04	0.25 ± 0.03	17 ± 8	17 ± 1	0.19 ± 0.11‡	0.20 ± 0.06‡

* Male mice of the C57Bl/6J and DBA/2J strains received phenobarbital (100 mg/kg, i.p.) in 0.9% saline. Controls received an equivalent amount of saline. The animals were sacrificed at the indicated intervals and the livers removed, chilled and weighed. Determinations of the activity ratio of cyclic AMP-dependent protein kinase, ornithine decarboxylase activity and RNA polymerase I activity were performed on samples from the same liver. The values represent the mean ± S. E. M. for duplicate determinations on three animals.

† Data differ from control ($P < 0.05$).

‡ Data differ from control ($P < 0.01$).

single doses of 3-methylcholanthrene, phenobarbital, polychlorinated biphenyls, or DDT to rats results in a significant elevation of the activity ratio of the hepatic cAMP-dependent protein kinase(s) [10–12, 35]. In fact, in response to the administration of Aroclor 1254 (a polychlorinated biphenyl), a low dose of 50 mg/kg, which in itself does not induce an increased microsomal concentration of P-450, together with either aminophylline, a phosphodiesterase inhibitor, or dibutyryl cAMP results in significant induction of cytochrome P-450 [11]. Yamasaki *et al.* [36] have also reported that the addition of dibutyryl cAMP and/or aminophylline to isolated baby hamster cells results in the induction of aryl hydrocarbon hydroxylase.

The inbred strains of mice characterized by an altered receptor protein having a diminished affinity for polycyclic hydrocarbons provide excellent models to delineate the specific biochemical events that occur after drug administration and which are prerequisite to enzyme induction. Increased hepatic cAMP concentration, as well as the activation of cAMP-dependent protein kinase, occurred early after the administration of 3-methylcholanthrene to the aryl hydrocarbon responsive mice whereas the aryl hydrocarbon nonresponsive mice exhibited no increase in hepatic cAMP concentration nor any significant activation of cAMP-dependent protein kinase in response to 3-methylcholanthrene. That these mice were capable of responding to other drugs is demonstrated by the use of phenobarbital which induces the hepatic oxygenase ethylmorphine *N*-demethylase to similar levels in both strains. After phenobarbital, there was rapid activation of cAMP-dependent protein kinase, induction of ornithine decarboxylase, and activation of RNA polymerase I followed by both the specific induction of ethylmorphine *N*-demethylase and significant hepatic hypertrophy. It is important to stress that drug administration which did not result in the activation of cAMP-dependent protein kinase (that is, 3-methylcholanthrene administered to aryl hydrocarbon nonresponsive mice) did not result in any detectable increases in ornithine decarboxylase activity, RNA polymerase I activity, aryl hydrocarbon hydroxylase activity, or hypertrophy.

These data lend further credence to the model of hypertrophy proposed in Fig. 1. Future studies should explore alternative models in attempts to understand the complexities of induction of the microsomal drug-metabolizing enzymes and associated hypertrophy.

Table 4. Changes in hepatic ethylmorphine *N*-demethylase activity in C57Bl/6J and DBA/2J mice after the administration of phenobarbital*

Strain	Ethylmorphine *N*-demethylase activity (nmoles HCHO/mg microsomal protein/10 min)	
	Control	Phenobarbital
C57Bl/6J	48.8 ± 3.1	76.9 ± 1.9†
DBA/2J	48.6 ± 1.2	82.3 ± 3.6†

* Male mice (20–25 g) of the C57Bl/6J and DBA/2J strains received phenobarbital (100 mg/kg, i.p.) daily for 3 days. On day, 5, the animals were sacrificed and the livers assayed for ethylmorphine *N*-demethylase activity. The values are the means ± S. E. M. for seven animals. Control animals received the injection vehicle alone.

† Data differ from control ($P < 0.001$).

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