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STIMULATION BY HEMATIN OF MONOOXYGENASE ACTIVITY IN EXTRA-HEPATIC TISSUES FROM RATS, RABBITS AND CHICKENS

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Summary: Micromolar quantities of hematin, when added to reaction flasks containing homogenates of extra-hepatic tissues, benzo(a)pyrene, and cofactors necessary for monooxygenation, caused up to a 40-fold enhancement of aryl hydrocarbon (benzo(a)pyrene) hydroxylase (EC 1.14.14.2) activity. Pretreatment of rats with 3-methylcholanthrene caused a reduction in the stimulatory effect of hematin. Metabolic profiles of 7,12-dimethylbenz(a)-anthracene, obtained with high pressure liquid chromatography, illustrated the enhancement of monooxygenase activity by hematin at methyl carbons. It is speculated that the mechanism of the hematin effect is the reconstitution of a small pool, or pools, of apo-(cytochrome P-450) which exist in these extra-hepatic tissues, resulting in the formation of functional holoenzyme.

Introduction: The regulation of holo-(cytochrome P-450) pools in animal tissues appears to be subject to coordinated controls involving hematin biosynthesis in the mitochondria, apo-(cytochrome P-450) synthesis in the rough endoplasmic reticulum and finally, the incorporation of hematin into the apoprotein yielding a functional holoenzyme (1, 2). However, the control mechanisms regulating this coordinated biosynthesis are not well elucidated. Recent results from this laboratory have demonstrated that additions of hematin *in vitro* caused a marked increase (25-30-fold) in aortic monooxygenase activity of the rabbit (3). The mechanism of the observed increase, however, was not investigated.

Here we report the finding that micromolar quantities of hematin, when added *in vitro*, markedly enhance the monooxygenase activity of several extra-hepatic tissues. The phenomenon was examined with regard to tissue, species and substrate specificity, concentration-effect relationships, and monooxy-

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genase induction with 3-MC[1].

Materials and Methods: [7-10-^{14}C]BaP (specific activity: 60.7 mCi/mmole, 97% pure by TLC on silica gel in hexane fraction) and [^{14}C]DMBA with a specific activity of 21.2 mCi/mmole (95% purity by TLC on silica gel in hexane fraction) were obtained from Amersham/Searle, Arlington Heights, IL. Hematin was obtained from Calbiochem, Los Angeles, CA; 3-MC from Mann Research Labs, Inc., New York, NY; BaP from Eastman Kodak Co., Rochester, NY; SDS from Bio-Rad Labs, Richmond, CA; and Aquasol from New England Nuclear, Boston, MA. NADPH, NADH, G6PDH, G6P and BSA were obtained from Sigma Chemical Co., St. Louis, MO. All other reagents and chemicals utilized were of the highest purity commercially obtainable.

Sprague-Dawley male rats, weighing approximately 200 gms were purchased from Tyler Labs, Inc., Bellevue, WA. New Zealand white male rabbits weighing approximately 6 lbs were purchased from Totem Farms, Kirkland, WA. White Leghorn chickens, 18-22 weeks of age, were purchased from Heisdorf and Nelson Co., Redmond, WA.

All tissues were pooled and homogenized at 4°C in 3 volumes of 0.1 M potassium phosphate buffer, pH 7.35, using a teflon homogenizer except for lung and aorta, which were homogenized with a Polytron PT-10 homogenizer. The homogenates were then spun at 9,000 x g for 5-20 min and the supernatants collected and frozen at -80°C until the assays were performed.

Formation of phenolic metabolites by tissue homogenates was assayed with [7-10-^{14}C]BaP according to the method of Brown and Kupfer (4), slightly modified as described below. (Earlier observations by these workers have shown that the presence of heme in the incubation mixtures was responsible for non-reproducible results when the standard fluorometric assay (5) was employed. This phenomenon was attributed to the finding that heme quenches the fluorescence of the 3-hydroxy metabolite of BaP.)

9,000 x g supernatant fractions were incubated in scintillation vials shaking in a Dubnoff incubator at 37°C for 2 hours at 100% oxygen tension. Reaction vessels were assayed in triplicate and typically contained final concentrations of the following: [7-10-^{14}C]BaP (150,000 dpm), BaP (40 nmoles), NADPH (1.10 x 10^{-3}M), NADH (7.0 x 10^{-4}M), G6P (2.5 x 10^{-3}M), hematin (0-24 μM) and sufficient potassium phosphate buffer (0.1 M, pH 7.35) to bring the total volume to 1.0 ml. Blanks contained heat-inactivated (100°, 5 min) enzyme. After extraction, the samples were neutralized with HCl and counted in a Beckman LS 9000 liquid scintillation counter with an efficiency greater than 85%. All samples were counted for a sufficient length of time to insure <7% error at a 95% confidence interval. Livers were assayed identically except for the addition of 2 units per flask of G6PDH. All experiments were performed in darkened conditions under yellow lights. Protein was assayed using BSA as the standard according to the method of Lowry *et al.* (6).

Specific activities are expressed as picomoles products formed/mg protein/flask/2 hr incubation and were calculated according to the formula:

$$\frac{\text{DPM recovered in NaOH phase x 40 nmoles}}{\text{Total DPM added x mg protein/flask}}$$

[1]Abbreviations used: 3-MC, 3-methylcholanthrene; BaP, benzo(a)pyrene; DMBA, 7,12-dimethylbenz(a)anthracene; SDS, sodium dodecyl sulfate; G6PDH, glucose-6-phosphate dehydrogenase; G6P, glucose-6-phosphate; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; BSA, bovine serum albumin; HPLC, high-pressure liquid chromatography.

Total radioactivity recovered in the organic and aqueous (NaOH) phases was 97 ± 2% of that added to the reaction vessels.

HPLC analysis of the metabolism of DMBA was performed according to methods described previously (7). Spectral analyses were performed with a model DW-2 recording spectrophotometer (American Instrument Co.) and difference spectra were monitored in the split-beam mode. Due to the spectral interference of hematin on cytochrome P-450 measurements, a system was developed to allow accurate spectral measurements. Sample and reference cuvettes each contained 9,000 x g supernatant, buffer and dithionite. Concentrations of P-450 were determined by selectively degrading the P-450 in the reference cuvette by addition of SDS. This procedure gave results identical to the standard method of Omura and Sato (8) while resolving the problem of hematin interference since we then could balance the hematin in each cuvette following incubation of the tissue homogenates.

Results: Addition of hematin to reaction vessels stimulated the monooxygenase activity in every extra-hepatic tissue examined. Although we always observed the stimulatory effect of hematin in these tissues, the magnitude of this effect and the basal levels of monooxygenase activity varied from one tissue preparation to another.

Hematin concentrations employed in these experiments were those that were found to be maximally effective for each tissue. As is shown in Fig. 1, additions of 1 to 10 μM (final concentration) hematin caused a sharp increase in monooxygenase activity which proceeded to plateau at slightly higher concentrations.

Table 1 depicts the monooxygenase activities, with and without hematin, for several hepatic and extra-hepatic tissues from three animal species. The response was most pronounced in extra-hepatic tissues of rabbits with the testes exhibiting the highest observable stimulation (41x). Interestingly, hematin inhibited the hydroxylating activity of liver after a 2 hour incubation. After incubations of 15 minutes, however, this effect was not seen. Indeed, under these conditions, hematin caused a slight increase in the activity of the hepatic enzyme.

In Table 2, the effect of induction by 3-MC on hematin stimulation is depicted. As can be seen, the tissues which responded the most dramatically to 3-MC also showed a marked decrease in their response to hematin. The testes, which exhibited only a 2-fold induction as a consequence of 3-MC

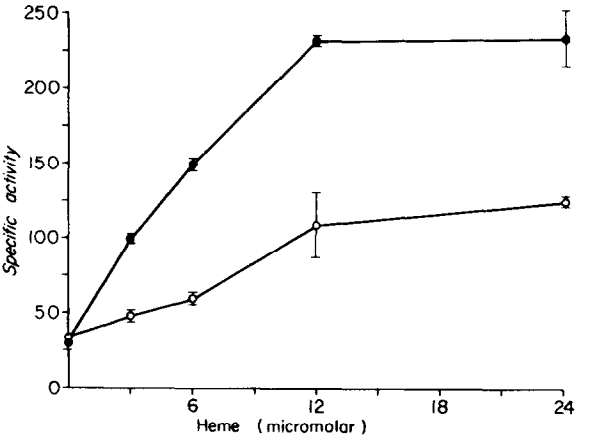

Figure 1. Effect of increasing hematin concentration on microsomal monooxygenase activity in rat lung (0) and rat kidney (●).

TABLE 1

Tissue	Specific Activity No Heme	Specific Activity + Heme	Ratio
RAT:			
Kidney	89 ± 7.8	406 ± 86	4.6
Testes	100 ± 3.4	194 ± 20	1.9
Lung	24 ± 0.7	134 ± 12	5.7
Liver	4326 ± 521	3351 ± 116	0.8
RABBIT:			
Kidney	85 ± 2	1432 ± 198	16.9
Testes	41 ± 10	1681 ± 193	41.0
Aorta	41 ± 13	1126 ± 40	27.5
Liver	1490 ± 66	1206 ± 49	0.8
CHICKEN:			
Testes	34 ± 21	704 ± 109	21.0
Aorta[a]	1.7 ± 0.5	26 ± 1.0	15.6
Liver	5169 ± 26	4459 ± 84	0.9

Specific Activities are expressed as picomoles product formed/mg protein/2 hr. All values represent means ± S.D.

[a]0.5 ml of aortic whole homogenate was incubated for 1 hour. Activity is expressed as picomoles/mg protein/hr.

TABLE 2

Rat Tissue	Controls Specific Activities			3-MC Pretreated[a] Specific Activities		
	No Heme	+ Heme[b]	Ratio	No Heme	+ Heme	Ratio
Kidney	29 ± 0.4	234 ± 18.2	8.1	483 ± 21	821 ± 42	1.7
Testes	22 ± 2.8	135 ± 26	6.1	53 ± 5.2	137 ± 5.6	2.6
Lung	34 ± 5.4	125 ± 3.4	3.7	211 ± 14	221 ± 9.7	1.1

[a]Single intraperitoneal injections of 3-MC (80 mg/kg) were given 48 hours before sacrifice.

[b]Heme was added in a final concentration of 24 μM in all experiments.

treatment, did not exhibit the same order of decrease in hematin stimulation.

The generalized nature of the effect of hematin on monooxygenase activity in extra-hepatic tissues is rather remarkable. Our data indicated that tissues possessing low basal levels of activity exhibited the highest response to hematin-mediated stimulation.

Our attempts to detect a change in P-450 concentrations in those tissues incubated with hematin (see Methods) were negative in that no spectrally detectable increase in cytochrome P-450 concentrations could be noted, even in tissue preparations in which stimulation of monooxygenase activity by hematin was highly pronounced (e.g., rabbit testes).

HPLC analysis of DMBA metabolism in the rabbit aorta (Fig. 2) showed that the 7-hydroxymethyl metabolite of DMBA was significantly increased when the incubation was carried out in the presence of hematin. No other alteration in the metabolic profile for DMBA could be detected.

Discussion: Using rat hepatic microsomes, investigators have demonstrated a slight enhancement of BaP-hydroxylase (4) and ethylmorphine N-demethylase (1) activities along with the hematin-mediated reconstitution of hepatic apo-(cytochrome P-450) (1, 9). In addition, Bissell and Hammaker (10) have presented findings suggesting that the heme moiety of cytochrome P-450 dissociates from its apoprotein and, prior to its degradation, mixes with endogenously

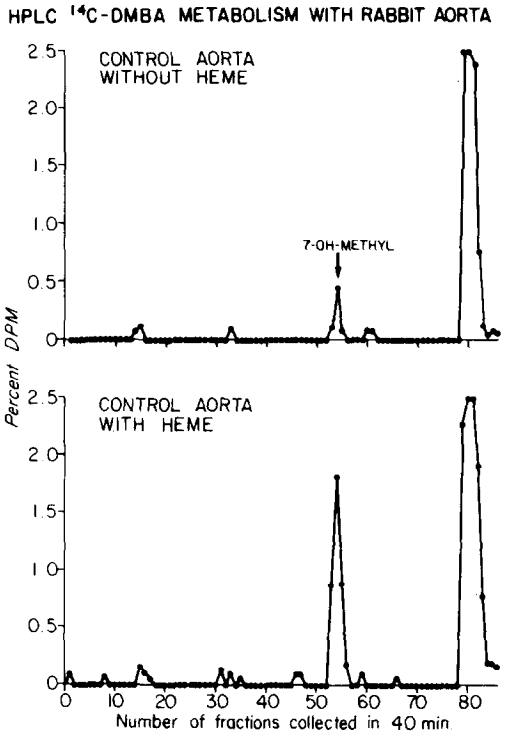

Figure 2. High-pressure liquid chromatographs of DMBA metabolites formed in incubation flasks containing homogenates of rabbit aortas. Radioactivity appearing in profiles from flasks containing heat-inactivated (100°, 5 min) homogenates has been subtracted.

synthesized heme to form a pool that regulates δ-aminolevulinic acid synthetase activity. Using SDS-gel electrophoresis, Siekevitz (11) has provided evidence for the existence of apo-(cytochrome P-450) in fetal rat liver microsomes, which, during late fetal development, binds heme and forms a complete holoenzyme molecule.

In attempts to monitor the cytochrome P-450 levels with time, we were unable to spectrally detect a concomitant increase of cytochrome P-450 concentrations in those tissues incubated in the presence of hematin. However, these data do not rule out the possible existence, in these tissues, of only a very minor pool, or pools, of apo-(cytochrome P-450), subject to reconstitution with the addition of exogenous hematin. Indeed, this would be consistent with other observations from this laboratory demonstrating that aryl hydrocarbon

hydroxylase induction in the human placenta is not associated with increased levels of spectrally observable cytochrome P-450 (12). Additional support for this idea was given by Lucier *et al.* (13), who demonstrated that administration of TCDD to pregnant rats caused an increase in fetal liver BaP-hydroxylase activity while the cytochrome P-450 content remained unchanged.

Thus, the data presented here could be explained by the hypothesis that, in extra-hepatic tissues, pools of both apo- and holo-(cytochrome P-450) exist, and that upon incubation with hematin, a functional reconstitution of the apoprotein occurs which is in turn responsible for the enhanced monooxygenase activity. However, at this point, alternative explanations for the data presented here cannot be excluded. Further experiments are necessary to clarify the actual mechanism of the observed increases in monooxygenase activity.

The ability of hematin to enhance this activity in extra-hepatic tissues may prove to be a useful experimental tool in studying both the regulatory aspects of holoenzyme formation and in the study of monooxygenase activity in tissues which normally have only a very low capacity for monooxygenation.

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