DIFFERENCES IN P-420 HEMOPROTEINS FROM UN-TREATED AND 3-METHYLCHOLANTHRENE-TREATED RATS

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The administration of 3-methylcholanthrene to rats leads to changes in the enzyme systems of the liver which oxidize drugs. These changes have been related to changes in some of the spectral properties of microsomal cytochrome P-450. The altered cytochrome appearing after 3-methylcholanthrene treatment has been called P-450, P-448, P-446, P-450, and high-spin P-450 by different investigators. Among the questions which have been raised are whether there is more than one molecular species of cytochrome P-450 in hepatic microsomes from untreated animals and how 3-methylcholanthrene exerts its effect—by altering the relative amounts of existing cytochromes or by inducing the formation of a new cytochrome. As cytochrome P-420 is a soluble degradation product of cytochrome P-450, it was felt that changes in cytochrome P-450 induced by 3-methylcholanthrene might be reflected in cytochrome P-420 isolated from treated animals.

This work was supported by U.S. Public Health Service Grant GM 15477. A detailed report of the work will be published in another journal.

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3 Throughout this communication cytochrome P-450 is considered to be the P-450 hemoprotein found predominantly in livers of untreated rats; cytochrome P-448 (also known as cytochrome P-448) is considered to be the P-450 hemoprotein found predominantly in livers of 3-methylcholanthrene-treated rats. Cytochrome P-420 is derived from cytochrome P-450; deoxy-cytochrome P-420, from cytochrome P-450.

Liver microsomes from untreated rats or from rats which were sacrificed 20 hr after the last of four ip injections of 3-methylcholanthrene (20 mg/kg/day) were treated with 0.07% steapsin at 4°C for 24 hr under nitrogen. About 25% of the cytochrome P-450 was converted to cytochrome P-420 and released into solution (table 1). When the solution was desalted over Sephadex G-25 and concentrated to 1/4 of its original volume, cytochrome P-420 precipitated. Whereas in the case of untreated animals, the precipitated cytochrome P-420 is in the form of microtubules, no unique structure is seen in the precipitate of cytochrome P-420 from animals treated with 3-methylcholanthrene (I). Sucrose gradient fractionation resulted in some additional purification of the precipitates, but recoveries were poor.

Drug binding spectra of the aggregated cytochrome P-420 from untreated and 3-methylcholanthrene-treated rats are shown in fig. 1. Whereas tubules prepared from microsomes of untreated animals evoke both type I (hexobarbital) and type II (aniline) spectral shifts, the precipitated cytochrome P-420 from 3-methylcholanthrene-treated rats evokes only the type II spectrum. The addition of 1.6 mM hexobarbital or 10 mM aniline to soluble cytochrome P-420 did not cause spectral changes.

In the course of these studies it was noted that the calculated amount of cytochrome P-420 in the preparations from animals treated with 3-methylcholanthrene, using a millimolar extinction coefficient of 111 for the CO complex of the reduced pigment with a maximum at 420 nm, was greater than the content of heme as measured by a pyridine hemochromogen method. Since the preparations had no effect on the assay of added hemin chloride over the range of concentrations encountered, the reduced cytochrome-carbon monoxide complex of the material from 3-methylcholanthrene-treated rats had to have a millimo-
TABLE 1

<table>
<thead>
<tr>
<th>Source of Micromesomes</th>
<th>Recovery of P-420(^\circ)</th>
<th>Recovery of P-420(^\circ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>% nmol/mg protein</td>
<td>% nmol/mg protein</td>
<td></td>
</tr>
<tr>
<td>Supernatant after steep-</td>
<td>43</td>
<td>53</td>
</tr>
<tr>
<td>sin treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eluate from Sephadex</td>
<td>43</td>
<td>1.9</td>
</tr>
<tr>
<td>G-25</td>
<td>35</td>
<td>0.41</td>
</tr>
<tr>
<td>Aggregated P-420 hemoprotein</td>
<td>6.7</td>
<td>10.3</td>
</tr>
<tr>
<td>P-420 hemoprotein from</td>
<td>2.2</td>
<td>7.6</td>
</tr>
<tr>
<td>sucrose gradient fractionation</td>
<td>1.4</td>
<td>9.8</td>
</tr>
</tbody>
</table>

**nmol of P-420 hemoprotein in the fraction**

**nmol of P-420 hemoprotein in unfractioned microsomes**

The amount of P-420 hemoprotein from microsomes from 200 g of wet liver from untreated and MC-treated rats was 2520 and 3560 nmol, respectively. Calculations of the P-420 hemoprotein content of microsomes from both untreated and 3-methylcholanthrene-treated rats were based on an extinction coefficient of 110 mM\(^{-1}\) cm\(^{-1}\) (9). Values for P-420 from microsomes from rats treated with 3-methylcholanthrene can be recalculated to accommodate the assumption that P-420 hemoprotein from these microsomes is P-420 by substituting an extinction coefficient of 114 for 110 (see text).

**Fig. 1.** Drug binding to aggregated P-420 hemoproteins from hepatic microsomes from untreated (N) and 3-methylcholanthrene (MC)-treated rats. Micromesomes from a given source were placed in reference and sample cuvets (1 mg of protein per ml) and balanced in a dual beam spectrophotometer. Spectra were recorded after addition of saturating amounts of the type I compound, hexobarbital (final concentration 1.6 mM), or the type II compound, aniline (final concentration 10 mM), to the sample cuvet. The aggregated cytochrome P-420 preparation contained 7.7 nmol and the aggregated cytochrome P-420 preparation contained 8.1 nmol of P-420 hemoprotein per mg of protein based on an extinction coefficient of 110 mM\(^{-1}\) cm\(^{-1}\).

**Fig. 2.** Analytical scale disc electrophoresis of P-420 hemoproteins. Aggregated P-420 hemoprotein preparations from the livers of untreated (N) and 3-methylcholanthrene (MC)-treated rats were dissolved in 8M urea. Duplicate samples of each preparation as well as mixtures of the two (MC + N) were subjected to disc electrophoresis. One of the gels was stained to reveal proteins, the other to reveal heme. The diagram is a composite representation of bands revealed by both staining procedures.
cytochrome P450 in the presence of cytochrome P450. The failure to observe cytochrome P420 in preparations of microsomes from untreated animals subjected to electrophoresis (fig. 2) is not conclusive evidence for the absence of cytochrome P450 in the microsomes from which the preparation was derived because of the great loss of P420 hemoprotein incurred during the process (table 2). Cytochrome P450 (P-448) has been equated with high-spin cytochrome P450 (6, 7) and with type a cytochrome P450 (8).

High-spin cytochrome P450 can result from a variety of factors in structures as complex as microsomes, but its simplest expression relative to hepatic cytochrome P450 can probably be seen in cytochrome P450cam, which has been isolated in pure form from Pseudomonas putida grown in a medium containing camphor as the sole source of carbon (9). It exhibits a high-spin spectrum in its substrate-complexed state (10). It is not unlikely that cytochrome P450 may combine with membrane components that impart high-spin characteristics. The administration of polycyclic hydrocarbons increases the amount of high-spin P450 hemoprotein in microsomes (6, 7). This could occur in one or several ways: a) Cytochrome P450 in its free state is a high-spin cytochrome P450. Direct evidence for this is lacking. On the contrary, the absorption spectrum of solubilized cytochrome P450 does not show a peak at about 650 nm (11), which might be expected if cytochrome P450 were a high-spin cytochrome (7, 12). b) As demonstrated in this study and by others (5, 8, 13), residual amounts of administered polycyclic hydrocarbons remain attached to microsomes. It is likely that these residues or their metabolites combine with cytochrome P450, or with residual cytochrome P450 in microsomes from animals treated with polycyclic hydrocarbons, to produce the high-spin P450 hemoprotein complex.

**TABLE 2**

<table>
<thead>
<tr>
<th>Kind of Cytochrome</th>
<th>P420 Hemoprotein Applied to Column</th>
<th>P420 Hemoprotein Recovered from Eluate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Protein (mg)</td>
<td>Cytochrome (nmol)</td>
</tr>
<tr>
<td>P-420</td>
<td>20</td>
<td>105</td>
</tr>
<tr>
<td>P-450</td>
<td>39</td>
<td>140</td>
</tr>
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</table>

**Fig. 3.** Preparative scale disc electrophoresis of cytochrome P420.

Aggregated cytochrome P420 from livers of untreated rats was dissolved in 8 M urea and subjected to disc electrophoresis as described in the text. The apparatus was flushed at a rate of 1 ml/min and the eluate passed through an ultraviolet flow monitor into a fraction collector. The output of the flow monitor (— ) and the magnitude of the carbon monoxide difference spectrum (----) of the corresponding fraction are plotted as a function of time.

Twenty hours after the last of four daily intraperitoneal doses of tritium-labeled 3-methylcholanthrene (20 mg/kg/day), the amount of methylcholanthrene present in the microsomes was found to be only 16% of the amount of cytochrome P450 on a molar basis. This is consistent with the studies of Flesher (5) who found a similar amount of tritium-labeled dimethylbenzanthracene 24 hr after a single dose of 30 mg. Some of the label is solubilized from microsomes by steapsin treatment, and 40% of that solubilized is not retained on Sephadex G-25 during desalting. None of the label remains with the aggregated cytochrome P420, suggesting that the label is bound to another microsomal component. Gas chromatographic analysis of 3-methylcholeanthrene accounted for all of the label present in microsomes, thus ruling out the role of a metabolite.

The question of whether cytochrome P450 is a normal constituent of hepatic microsomes, whether it appears only in response to compounds such as polycyclic hydrocarbons, or whether it exists normally in very small quantities that can be greatly increased when induced by the administration of these foreign compounds, is difficult to answer because of the lack of specificity and sensitivity of the methods used to measure
DIFFERENCES IN CYTOCHROMES P-420 AND P-450

Type a cytochrome P-450 has been described as one of two forms of P-450 hemoprotein distinguishable by differences in the type II binding spectra they produce when complexed with octylamine and other primary amines, namely type a with \( \lambda_{\text{max}} = 427 \) nm, \( \lambda_{\text{min}} = 392 \) nm, and type b, with \( \lambda_{\text{max}} = 432 \) nm, \( \lambda_{\text{min}} = 410 \) nm (8). The type a form is increased in microsomes from animals treated with 3-methylcholanthrene. In addition to the possibility that the two "forms" of cytochrome P-450 may simply represent two binding sites on a single cytochrome P-450 molecule, the alternative explanation must be considered that both cytochromes P-450 and P-450 may exist in two forms (or possess two amine binding sites), and that cytochrome P-450 exists to a higher degree in the type a form than does cytochrome P-450. If one accepts that cytochrome P-450 is one of two naturally occurring forms of P-450 hemoprotein and that of the two forms, it has the highest propensity for type a binding, it follows that microsomes from untreated animals will necessarily contain a considerable amount of cytochrome P-450, estimated by Jefcoate and Gaylor (7) to be 25% of the P-450 hemoprotein. It is of interest that these investigators detected electron paramagnetic resonance at \( g = 6.6 \) only in microsomes from 3-methylcholanthrene-treated animals. As the specific content of the high-spin form in the phenobarbital-induced preparation is calculated to be 1.5 times that in the 3-methylcholanthrene-induced preparation, it seems unlikely that type a cytochrome P-450 is indeed a high-spin hemoprotein. The high-spin signal probably represents the effect of residual 3-methylcholanthrene in the microsomes.

Much evidence has accumulated to show that cytochrome P-450 is not simply a relatively stable complex of cytochrome P-450 with 3-methylcholanthrene or one or more of its metabolic derivatives (14). One of the more convincing arguments supporting this view was the observation that purified, soluble cytochrome P-450 from rats injected with tritiated 3-methylcholanthrene contained radioactivity equivalent to only 0.04 mol of 3-methylcholanthrene per mol of P-450 hemoprotein (11). However, the possibility remained that even this small amount of the polycyclic hydrocarbon in combination with cytochrome P-450 could have accounted for the small differences seen in spectral characteristics of the imiure cytochrome P-450 and P-450 preparations. The current finding that cytochrome P-450 from animals injected with tritiated 3-methylcholanthrene is devoid of radioactivity, while still differing from cytochrome P-420 in its electrophoretic migration, binding to drugs, and spectral extinction coefficient, contributes strongly to the previous conclusion that cytochrome P-450 is a distinct chemical entity not to be equated with a complex of polycyclic hydrocarbon and cytochrome P-450.

References

Discussion

Dr. Nebert, NICHHD: During this symposium I hope the dogma will be laid to rest that in vivo administration of 3-methylcholanthrene per se or the metabolites of in vivo administered aromatic hydrocarbons per se, cannot be responsible for the increase in type a cytochrome P-450 [C. R. E. Jefcoate, R. L. Calabrese, and J. L. Gaylor, Mol. Pharmacol., 6, 391 (1970)]. The reasoning for this is as follows. Certain genetically dif-
ferent inbred strains of mice and hybrids from appropriate genetic crosses do not respond to in vivo aromatic hydrocarbon treatment [D. W. Nebert, F. M. Goujon, and J. E. Gielen, Nature New Biol. 236, 107 (1972); J. E. Gielen, F. M. Goujon, and D. W. Nebert, J. Biol. Chem., 247, 1125 (1972)] although the polycyclic hydrocarbon presumably binds to cytochrome P-450 and is actively metabolized in the liver and in nonhepatic tissues. On the contrary, inbred, random bred, or hybrid mice have at least one dominant Ah allele which responds to the aromatic hydrocarbon treatment in vivo. As will be described tomorrow in my report, this "response" includes 1) a blue spectral shift in the Soret peak of the reduced P-450-CO complex, 2) increases in the specific activities of several mixed function oxygenases, 3) an increase in the type a/type b forms of P-450 as measured by room temperature spectrophotometry, and 4) an increase primarily in the high-spin iron species of cytochrome P-450 detectable by electron paramagnetic resonance spectroscopy below 10°K.

Dr. Raisfeld, New York U.: The material which you showed on the electron micrograph resembles fragments of membranes. Have you been able to perform electrophoresis with this material in anything other than 8 M urea?

Dr. Shoeman: No, no other medium. Let me say that there weren't any successful electrophoretic experiments done in anything other than 8 M urea.