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Induction and Tissue-Specific Expression of Rabbit Cytochrome P450IIE1 and IIE2 Genes

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SUMMARY

Treatment of rabbits with a variety of dissimilar chemicals, including ethanol, acetone, and imidazole, results in elevated levels of hepatic and renal cytochrome P-450 form 3a, also designated P-450$_{ALC}$ or P-450IIE. The P450IIE1 subfamily in rabbits is composed of two genes that encode proteins with 97% sequence identity; the mRNAs from these genes can be distinguished by their differing electrophoretic mobilities. In the present studies, examination of the expression of these genes revealed that P450IIE1 (gene 1) mRNA is present in greatest abundance in the liver, is present in kidney and nasal mucosa at approximately 10% of the level in liver, and is present in lung at approximately 5% of the level in liver. P450IIE2 (gene 2) mRNA is present in liver and lung at approximately 50% of the level of gene 1 mRNA in these tissues but cannot be detected in kidney or nasal mucosa. Neither gene is expressed in testis, ovary, small intestine, or adrenal tissue. Treatment of rabbits with acetone or imidazole results in elevated levels of P-450 3a-immunoreactive protein in liver and kidney without concomitant increases in P450IIE gene mRNAs. Moreover, various lengths of ethanol treatment elevated the level of immunoreactive protein in liver and kidney, with a rapid reduction of gene 1 mRNA and, at 14 days, gene 2 mRNA to approximately 50% of control levels. In contrast to these chemical inducers of 3a, fasting for 48 hr significantly increases gene 1 and 2 mRNA in liver but does not increase the level of immunoreactive protein. These results indicate that the rabbit P450IIE genes are not coordinately expressed or regulated and, as found with the rat ortholog P-450j, chemical inducers of 3a evidently act through changes in the rate of synthesis or degradation of the enzyme, rather than through increased gene transcription.

Cytochrome P-450 3a is the rabbit alcohol-inducible form (also designated P-450IIE1 or P-450$_{ALC}$) (1). This cytochrome is active in the metabolism of a variety of compounds such as alcohols and halogenated alkyl compounds, including many commonly encountered solvents, and in the oxidation of some nitrosamine carcinogens and acetaminophen to reactive intermediates (reviewed in Ref. 2). In rabbits the P450IIE subfamily is composed of two highly similar genes that encode cytochromes differing in primary structure by less than 3% (3). Gene 1 (P450IIE1) encodes P-450 3a, whereas gene 2 (P450IIE2) encodes a protein that has not yet been isolated and characterized.[1] The expression of isoform 3a and its rat ortholog, P-450j, has been examined previously in various tissues and after various treatments known to induce this enzyme or its catalytic activities. In rabbits the enzyme has been identified immunochemically in liver, kidney, and nasal mucosa (4, 5) and has been shown to be inducible in the liver by a variety of dissimilar chemicals such as acetone, imidazole, ethanol, pyrazole, and isoniazid (1, 5–7); the enzyme can be induced in the kidney by ethanol treatment (4, 5). Preliminary studies on the induction of P-450 3a with the use of a 3a cDNA probe indicated that imidazole treatment elevated the enzyme without increasing 3a mRNA (8). Similar results have been obtained with the rat; hepatic P-450j is elevated by a variety of chemical treatments (9–15) without a concomitant increase in P-450j mRNA (12–15). This nontranscriptional induction of P-450j by chemicals contrasts with induction by physiological regimens such as fasting and diabetes, where both P-450j enzyme (11, 14–20) and mRNA levels (14–17) are elevated.

The present studies were undertaken to investigate the tissue-specific expression and mechanism of induction of the two P450IIE genes in the rabbit. The results indicate that these genes are not coordinately expressed or regulated, and, as found with P-450j in the rat, chemical inducers of 3a evidently act through changes in the rate of synthesis or degradation of the enzyme, rather than through increased rates of gene transcription.

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[1] Because of the high degree of similarity between the two encoded proteins, our polyclonal and monoclonal antibodies do not differentiate between them, and, because the proteins differ in mass by only 100 daltons, they do not separate upon SDS-polyacrylamide gel electrophoresis; however, the protein encoded by the second gene has been demonstrated to be present in the liver by N-terminal sequence analysis (E. S. Roberts, X. Ding, and M. J. Coon, unpublished observations). In this paper, reference to the enzyme in liver, but not in kidney or nasal mucosa, necessarily includes both gene products.

ABBREVIATION: SDS, sodium dodecyl sulfate.

Experimental Procedures

Animal treatments. Male New Zealand white rabbits (2.0–2.5 kg; Langshaw Farms, Augusta, MI) were housed in metal cages with a 12-hr light-dark cycle and free access to rabbit chow and water. Treatments were as follows: untreated controls ($n = 6$); 0.1% actone in the drinking water for 7 days ($n = 6$); 200 mg/kg imidazole intraperitoneally 20 and 12 hr before death ($n = 3$); ethanol (2.5 g/kg) as a 25% solution in water via an orogastric tube 12 hr before death ($n = 3$); ethanol (2.5 g/kg) as a 25% solution in water via an orogastric tube 24 hr before death ($n = 3$); 10% ethanol in the drinking water for 7 days ($n = 3$); 10% ethanol in the drinking water for 14 days ($n = 3$); fasted for 48 hr ($n = 3$); and female untreated rabbits ($n = 3$). Blood ethanol levels in the acutely treated animals (ethanol 12 and 24 hr before death) were approximately 200 mg/100 ml at 1 hr after dosing and had returned to control levels by 12 hr after treatment, as determined with a blood alcohol assay kit (Sigma Chemical Co., St. Louis, MO). The rabbits were killed by barbiturate injection into an ear vein, and tissues (liver, lung, kidney, testis, ovary, adrenals, and approximately 10 cm of the proximal small intestine) were immediately removed, frozen in liquid N_2, and stored at −70°.

Preparation and analysis of RNA. Total RNA was prepared by the method of Chirgwin *et al.* (21) from 2.5 g of each tissue. The tissue samples were defrosted in the guanidinium isothiocyanate buffer and were homogenized with a Brinkmann Polytron. The isolated RNA was resuspended in diethylpyrocarbonate-treated water and quantified spectrally (1 A_{260} unit = 40 μg/ml). Poly(A)$^+$ RNA was isolated by oligo(dT) cellulose (Pharmacia, Piscataway, NJ) chromatography (22). Glyoxal-denatured pooled RNA samples were fractionated on 1% agarose gels, as described (23), and transferred to Zeta Probe membranes (Bio-Rad, Richmond, CA) by diffusion overnight. RNA was fixed to the membranes by baking at 80° under a vacuum for 2 hr, and the filters were prehybridized for 2 hr at 58° in 6× SSPE (0.9 M NaCl, 60 mM $NaPO_4$, and 6 mM Na_2EDTA, pH 7.4), 0.1% SDS, 10× Denhardt's solution without serum albumin (0.2% polyvinylpyrrolidone and 0.2% Ficoll 400), 50 μg/ml yeast RNA, and 50 μg/ml sheared denatured DNA. Hybridizations were carried out in 6× SSPE and 1% SDS for 12–18 hr at 58°, with 3–6 × 10^6 cpm/ml concentrations of an end-labeled (^{32}P) oligonucleotide complementary to genes 1 and 2 (3).[2] The filters were washed three times in 6× SSPE and 1% SDS at 58° and were exposed to X-ray film with an intensifying screen overnight. Appropriately exposed autoradiograms were scanned on a laser densitometer (Helena Laboratories, Beaumont, TX) for quantitation.

Preparation and analysis of microsomes. Microsomes were prepared from 2.5 g of liver and kidney (24), and the protein concentration was determined by the method of Lowry *et al.* (25). Pooled samples at five or more concentrations were transferred to nitrocellulose (Schleicher and Schuell, Keene, NH) with the use of a Hybri-Slot manifold (Bethesda Research Laboratories, Rockville, MD) and 3a-immunoreactive protein levels were determined by the method of Domin *et al.* (26) with the use of a monoclonal antibody to P-450 3a (4). Immunoconjugates were detected by autoradiography with an ^{125}I-labeled anti-mouse antibody (Amersham, Arlington Heights, IL) and were quantified by scanning on a laser densitometer. SDS-polyacrylamide gel electrophoresis of pooled microsomes was performed according to the method of Laemmli (27) in 7.5% gels, the fractionated protein was electroblotted to nitrocellulose (Bio-Rad), and 3a-immunoreactive protein was visualized with the monoclonal and radioiodinated antibodies (Amersham), as described above.

[2] An earlier study (8) revealed that the P-450 3a cDNA hybridized to multiple mRNA species when used to probe electrophoretically fractionated liver RNA. Although the 3′ nontranslated portion showed greater specificity, it does not hybridize to gene 2 mRNA due to a large deletion in the 3′ nontranslated portion of this gene (3). To avoid potential cross-hybridization of our cDNA to other unidentified RNAs, as suggested in the earlier study (8), we utilized a 20-base oligonucleotide complementary to nucleotides 110 to 119 of the P450IIE mRNAs (3).

Results

Tissue-specific expression. An oligonucleotide that specifically recognizes mRNA from both genes of the rabbit cytochrome P450IIE subfamily was used to screen for the expression of these genes in a variety of tissues. Only liver, kidney, nasal mucosa, and lung expressed P450IIE mRNA (Fig. 1). Testis, ovary, the proximal small intestine, and the adrenals did not exhibit measurable expression of either gene. Gene 2 mRNA was further limited to liver and lung, where it was present at about 50% of the level of gene 1 mRNA; it could not be detected in kidney or nasal mucosa even with prolonged autoradiographic exposures. Gene 1 (P-450 3a) expression was greatest in the liver, whereas its expression in kidney and nasal mucosa was 10% of that of liver and in lung it was present at only 4–5% of its level in liver. P-450 3a protein can be detected immunologically in liver, kidney, and nasal mucosa, but it is not detectable in the lung (4). Thus, based on these and earlier studies (3), the protein detected immunochemically in the liver consists of both gene 1 and gene 2 proteins, whereas the protein detected in kidney and nasal mucosa consists of only the gene 1 (P-450 3a) protein.

Effects of inducers on gene expression. The effect of several inducers of cytochrome P-450 3a on the level of gene 1 and gene 2 mRNA in liver and kidney was examined by using the radiolabeled oligonucleotide to probe electrophoretically fractionated RNA from variously treated animals, as shown in Fig. 2. The results of densitometric scans of these autoradiograms and two other experiments are compiled in Table 1. The effect of these inducers on the level of 3a-immunoreactive protein in liver and kidney is shown in Fig. 3, and average values as a percentage of the control protein level are included in Table 1. Of the four treatments examined, only fasting was able to significantly increase gene 1 and gene 2 mRNA levels in liver and kidney; acetone, the most effective inducer of P-450 3a (7), elevated mRNA levels in liver and kidney by less than 50%, while increasing 3a-immunoreactive protein by 5.6-fold in the liver and over 8-fold in the kidney. Surprisingly, imidazole and ethanol treatments decreased gene 1 mRNA by 28% and by as much as 57%, respectively; imidazole, nonetheless, produced a 4-fold increase in immunoreactive protein in the liver and a 6.7-fold increase in the kidney. The rapid reduction in gene 1 mRNA with ethanol treatment was noted

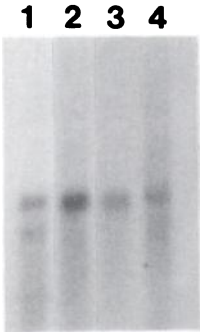

Fig. 1. Tissue-specific expression of P450IIE genes 1 and 2. Poly(A)$^+$ RNA was fractionated by agarose gel electrophoresis, transferred to Zeta Probe membranes, and hybridized to a radiolabeled oligonucleotide that recognizes mRNA from both genes, as described in Experimental Procedures. The *upper band* corresponds to gene 1 mRNA (P-450 3a); the *lower band* corresponds to gene 2 mRNA (3). *Lane 1*, 0.5 μg of liver RNA; *lane 2*, 5 μg of kidney RNA; *lane 3*, 5 μg of nasal mucosa RNA; *lane 4*, 10 μg of lung RNA.

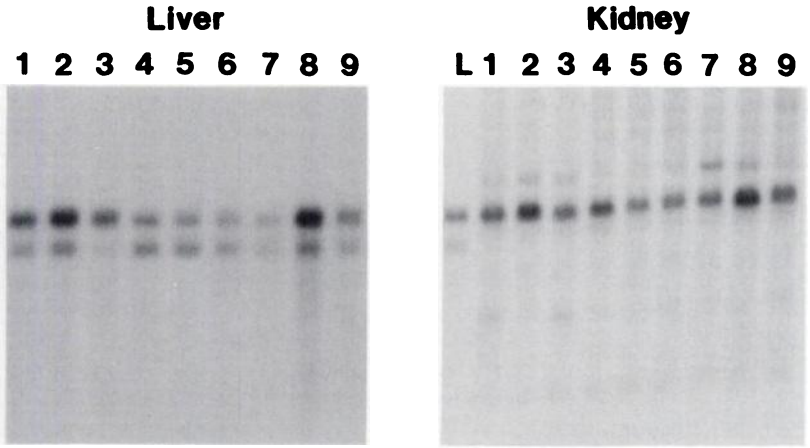

Fig. 2. Effects of P-450 3a inducers on the expression of genes 1 and 2 in liver and kidney. Pooled samples of total RNA were fractionated by agarose gel electrophoresis, transferred to Zeta Probe membranes, and hybridized to the radiolabeled oligonucleotide that recognizes mRNA from both genes, as described in Experimental Procedures. The *upper band* corresponds to gene 1 mRNA (P-450 3a); the *lower band* corresponds to gene 2 mRNA (3). Each liver sample contained 1 μg of RNA; each kidney sample contained 5 μg of RNA. *Lanes 1*, untreated; *lanes 2*, acetone-treated; *lanes 3*, imidazole-treated; *lanes 4*, 12-hr ethanol-treated; *lanes 5*, 24-hr ethanol-treated; *lanes 6*, 7-day ethanol-treated; *lanes 7*, 14-day ethanol-treated; *lanes 8*, fasted; *lanes 9*, female, untreated. *Lane L* in the kidney blot contained 1 μg of control liver RNA.

TABLE 1

Quantitation of products of genes 1 and 2 in liver and kidney

Treatments are described in Experimental Procedures. mRNA levels were quantitated by densitometry as described in Experimental Procedures and are expressed as the means and standard deviations of three determinations on pooled samples; protein levels were determined by immunoquantitation and densitometry as described in Experimental Procedures and are expressed as the average of two determinations on pooled samples.

Treatment	Liver			Kidney	
	Gene 1 mRNA	Gene 2 mRNA	Protein	Gene 1 mRNA	Protein
	% of control				
Control	100 ± 24	100 ± 24	100	100 ± 5	100
Acetone	139 ± 19	121 ± 36	561	149 ± 11[a]	819
Imidazole	72 ± 10	32 ± 11[a]	443	95 ± 9	671
Ethanol, 12 hr	50 ± 15[a]	125 ± 38	161	100 ± 16	176
Ethanol, 24 hr	45 ± 11[a]	115 ± 29	117	63 ± 11[a]	171
Ethanol, 7 days	43 ± 13[a]	103 ± 14	273	58 ± 16[a]	400
Ethanol, 14 days	43 ± 21[a]	69 ± 32	243	68 ± 19[a]	605
Fasting	303 ± 120	207 ± 41	124	146 ± 20	176
Female	90 ± 32	92 ± 20	45	90 ± 3	95

[a] Significantly different from control group, $p < 0.05$, by Dunnett's multiple comparisons test (two tailed), with fasted groups excluded due to unequal variances; too few measurements were made for a nonparametric test of these values. The effect of these treatments on protein levels also was not analyzed statistically, because only two measurements were made.

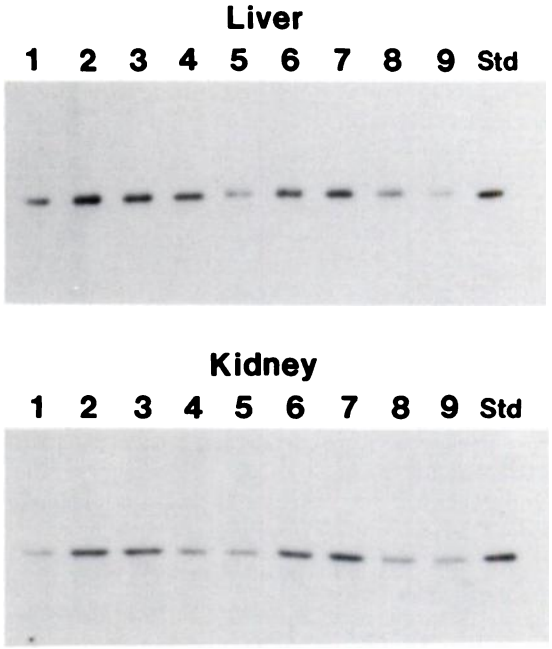

Fig. 3. Effects of inducers on P-450 3a-immunoreactive protein levels in liver and kidney. Pooled microsomes from liver (5 μg of protein) or kidney (25 μg of protein) were fractionated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with a monoclonal antibody to cytochrome P-450 3a (4), as described in Experimental Procedures. Lane designations are described in the legend to Fig. 2; *Std*, 100 ng of purified P-450 3a.

in liver as early as 12 hr after a single oral dose and was evidenced in the kidney at 24 hr after treatment; chronic ethanol treatment, while depressing mRNA levels to less than one half of control levels, produced a 2.5-fold increase in protein in the liver and up to a 6-fold increase in the kidney. Female rabbits expressed genes 1 and 2 at levels equivalent to those of males but exhibited less than one half the amount of immunoreactive protein in the liver.

Expression of gene 2 relative to gene 1. The effect of these inducers on gene 2 expression relative to gene 1 expression is illustrated in Table 2. Whereas the ratio of gene 2 mRNA to gene 1 mRNA in untreated rabbits was 0.53, this ratio was dramatically altered by imidazole and ethanol treatment and also by fasting. Imidazole and fasting decreased the ratio of gene 2 mRNA to gene 1 mRNA to 0.33 and 0.37, respectively; imidazole effected this decrease by causing a greater reduction of gene 2 mRNA (32% of control) than of gene 1 mRNA (72% of control), whereas fasting acted more strongly to increase gene 1 mRNA than gene 2 mRNA (303% versus 207% of controls, respectively) (Table 1). In contrast, ethanol treatments greatly increased the ratio of gene 2 mRNA to gene 1 mRNA, an effect that was noted as early as 12 hr after a single oral dose and lasted through 7 days of chronic

TABLE 2

Expression in liver of gene 2 mRNA relative to gene 1 expression

Treatments are described in Experimental Procedures. mRNA levels were quantitated by densitometry, as described in Experimental Procedures.

Treatment	Gene 2/Gene 1 mRNA Ratio
Control	0.53
Acetone	0.44
Imidazole	0.33
Ethanol, 12 hr	1.31
Ethanol, 24 hr	1.35
Ethanol, 7 days	1.25
Ethanol, 14 days	0.85
Fasting	0.37
Female	0.55

treatment (Table 2). The effect of ethanol was mediated largely through a 50% decrease in gene 1 mRNA, with a slight increase in gene 2 mRNA (at 12 hr and 24 hr; Table 1). Ethanol treatment did not decrease gene 2 mRNA until 14 days of treatment, at which point the ratio of gene 2 mRNA to gene 1 mRNA, 0.85, was still elevated over that of untreated animals.

Discussion

The expression of cytochrome P-450 3a in a variety of rabbit tissues has been examined previously in this laboratory and found to be restricted to liver, kidney, nasal mucosa, and esophageal mucosa (4).[3] The recent discovery that the rabbit contains two highly similar genes in the P450IIE subfamily, which encode proteins with greater than 97% identity in primary structure (3), raised the question of whether these genes exhibit tissue-specific expression and are perhaps differentially inducible. The present studies reveal that the expression of the two genes is not tightly coordinated; gene 2 expression is limited to the liver and lung, and the level of gene 2 mRNA is modulated differently from the level of gene 1 mRNA in response to various inducers of P-450 3a.

The most noticeable disparity in expression levels of genes 1 and 2 occurs after ethanol treatment, when, in the liver, the amount of gene 1 mRNA is reduced to one half of the control level within 12 hr of a single oral dose, whereas the amount of gene 2 mRNA is slightly increased at this time point. This rapid reduction in gene 1 mRNA suggests a direct effect of ethanol on either gene 1 transcription or mRNA stability but does not exclude the possibility that the 60% increase in 3a-immunoreactive protein also evident at this time point causes the rapid reduction in gene 1 mRNA levels. That the level of gene 2 mRNA is not similarly reduced with ethanol treatment suggests that an "ethanol response element" is not present in this closely related gene. This response element could either act to decrease the rate of transcription of gene 1 or be present on gene 1 mRNA to decrease the stability of the message in the presence of ethanol. As the mRNAs from genes 1 and 2 differ predominantly in their 3′ nontranslated segments (notably, gene 2 mRNA contains a 338-base pair deletion of nontranslated sequence present in gene 1) (3), the possibility that the response element resides in this segment of gene 1 is attractive. Alternatively, the 5′ flanking regions of genes 1 and 2 contain a segment from approximately −150 to −310 that exhibits considerable sequence divergence, in contrast to the segments preceding and following this region (3). It is possible that this divergent segment is associated with the differences in the expression of these two genes in the presence of ethanol. Unfortunately, our present data do not allow us to discriminate between an effect on transcription and an effect on mRNA stability. Finally, it is of interest to note that two studies that examined the effect of ethanol on P450IIE mRNA levels in other species have not uncovered a similar phenomenon. Kubota *et al.* (28) reported a 2.6-fold *elevation* of P450IIE1 liver mRNA after treatment of hamsters with 10% ethanol in the drinking water for 10 days, whereas Johansson *et al.* (15) reported no change in P-450j mRNA after rats were fed an ethanol-containing liquid diet for 20 days. Thus, no consistent effect of ethanol on P450IIE mRNA levels is apparent among the rabbit, rat, and hamster, and the reduction in gene 1 mRNA by ethanol does not appear to be an evolutionarily conserved feature.

It can be concluded from these studies that most inducers of P-450 3a increase enzyme levels either by enhancing the translational efficiency or by diminishing the rate of enzyme degradation. In this study, only fasting was able to increase mRNA levels, an effect previously noted in the rat (14–16). These results further suggest that the protein is subject to rapid modulation of its levels in response to chemical inducers. For example, the imidazole treatment used in this study (two doses, at 20 and 12 hr before death) was designed to establish a rapid, short term induction that would maximize the probability of finding elevated 3a mRNA levels if induction is mediated through increased gene transcription or mRNA stabilization. Surprisingly, the 4.4-fold increase in immunoreactive protein in the liver obtained with this treatment was equivalent to that otherwise obtained with more prolonged treatment protocols (e.g., Ref. 4), and, moreover, was accompanied by a measurable decrease in the amount of gene 1 and gene 2 mRNA. Similarly, as noted above, a single oral dose of ethanol produced a 60% increase in immunoreactive protein within 12 hr, with a return to the control level at 24 hr. These results support a rapid rate of turnover for P-450 3a in the liver; in contrast, the rate of turnover appears to be slower in the kidney, where a 70% increase in protein was still evident 24 hr after ethanol treatment. Consistent with a slower turnover in the kidney, the extent of induction by all treatments was greater in kidney than in liver. As an alternative explanation, however, it is possible that the gene 2 protein is not stabilized by those agents that stabilize, and therefore induce, P-450 3a, thereby lowering the extent of induction measured in the liver, where both proteins are present, relative to that measured in the kidney, where only the inducible 3a protein is expressed. This explanation would imply that inducers interact differently with the two enzymes despite their considerable structural similarity. Purification or heterologous expression of the gene 2 protein, both of which are currently under way in this laboratory, will facilitate the testing of this hypothesis.

Finally, it is worth noting that, despite a significant increase in mRNA levels in the liver after fasting, the amount of immunoreactive protein was essentially unchanged, suggesting that enzyme levels may be only loosely coupled to mRNA levels. Consistent with this hypothesis, female rabbits expressed gene 1 and gene 2 mRNAs at levels approximately equal to those of males but expressed less than half the amount of protein in liver. Overall, these results support the suggestion of Eliasson *et al.* (13) of a role for ligands in maintaining P-450j enzyme levels. Inducers, or their metabolites, may increase ligand levels, either by functioning as a ligand or by elevating an endogenous ligand, and, thereby, increase enzyme levels through stabilization; perhaps female rabbits contain lower levels of an endogenous ligand and, thus, maintain lower levels of enzyme. These possibilities remain to be explored.

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