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# Pretranslational Activation of Cytochrome P450IIE during Ketosis Induced by a High Fat Diet

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**SUMMARY**

Ethanol-inducible cytochrome P450 (P450IIE) is reported to be induced by ketosis. In the present study, the effects of a high fat diet on P450IIE induction and the relationship between ketone body concentration and P450IIE induction were studied by the following: 1) measurement of the activity of aniline hydroxylase, 2) immunoblot analysis for P450IIE protein, and 3) Northern blot analysis for P450IIE mRNA. The enzyme activities (aniline hydroxylase) in hepatic and renal microsomes were elevated about 2–3-fold by feeding with a high fat diet for 3 days. The increases in enzyme activities were also accompanied by 3-fold increases in immunoreactive P450IIE protein and its mRNA. In contrast, no differences were observed for the catalytic activities of *N*-alkoxyresorufin dealkylases or the amounts of immunoreactive P450IA and P450IIC, indicating a specific induction of P450IIE by high fat feeding. Furthermore, the increases in the levels of P450IIE mRNA correlated positively ($r = 0.73$) with plasma concentrations of acetoacetate and β-hydroxybutyrate but not with that of acetone, which induces P450IIE without changing its mRNA level. Our data thus indicated that P450IIE induction during the ketosis of a high fat feeding appears to be due to pretranslational activation and that is similar to the induction mechanism of fasted and diabetic animals.

The P450s are the microsomal mixed function oxidases responsible for the primary metabolism of a variety of foreign substances such as drugs, carcinogens, and environmental chemicals, as well as the metabolism of endogenous substances such as fatty acids, prostaglandins, and steroids. P450s exist in multiple forms with overlapping substrate specificities. Many of the multiple forms of P450s have been shown to be products of different genes that encode proteins with distinct molecular weights, electrophoretic mobility, spectral properties, primary structures, immunological reactivities, and substrate specificities. Recent study has shown that there are at least 10 P450 gene families, eight of which exist in mammalian systems (reviewed in Ref. 1). The biochemical and genetic classification of these enzymes was recently compiled to generate a more systematic nomenclature of P450s (2). Many of these P450 proteins are induced by their own substrates (1, 3).

P450IIE has been purified to homogeneity (4) and characterized with respect to its substrate specificity and induction mechanism (5). The enzyme is thought to have an important role in metabolism of both endogenous and exogenous compounds (5–11), such as acetone, ethanol, ether, $CCl_4$, benzene, pyrazole, pyridine, and water-soluble carcinogens such as certain nitrosamines. In addition to these well known properties, P450IIE can act as a gluconeogenic enzyme in the conversion of fat to carbohydrate (6, 12). Furthermore, the expression of P450IIE is under complex regulation. Its induction by a variety of structurally unrelated chemicals, including its substrates (5–10, 13), as well as by pathophysiological conditions such as fasting and diabetes (14, 15), has been reported. Having characterized the cDNA clones for both rat and human P450IIE (16), we have also demonstrated multiple types of regulation of P450IIE induction (16–19). However, because of apparent differences in the mechanism of P450IIE activation between acetone-treated animals and animals under ketotic conditions, such as fasting and diabetes (14, 16), and positive correlation between the degree of P450IIE induction and the levels of a ketone body, β-hydroxybutyrate (20), the current study was designed to further identify which ketone bodies correlate positively with the increases in P450IIE mRNA. In the earlier studies (14), the direct effects of exogenous acetoacetate and β-hydroxybutyrate on P450 induction were examined without success, with little accumulation of these ketone bodies, probably because of rapid breakdown. In order to circumvent this problem and to demonstrate a positive correlation between the levels of ketone bodies and P450IIE mRNA induction, we, as shown in this study, had to use different strategies to increase the levels of ketone bodies, by using a fat diet.

## Materials and Methods

**Reagents.** The random-primed labeling kit was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). GeneScreen

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**ABBREVIATIONS:** P450, cytochrome P450; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); SDS, sodium dodecyl sulfate; Kb, kilobases.

TABLE 1

**Effects of various treatments on aniline hydroxylase and alkoxyresorufin-O-dealkylases in rat hepatic microsomes**

Data are presented as means ± standard errors (four experiments for each group, repeated three times). Values in parentheses are percentage of control.

| Treatment | Aniline hydroxylase activity[a] | Pentoxyresorufin-O-depentylase activity[b] | Ethoxyresorufin-O-deethylase activity[b] |
|---|---|---|---|
| | *nmol/min/mg* | *pmol/min/mg* | *pmol/min/mg* |
| Control | 0.35 ± 0.06 (100) | 112.8 ± 24.5 (100) | 112.8 ± 10.8 (100) |
| High fat | 0.78 ± 0.21 (223)[c] | 120.5 ± 19.5 (107) | 128.7 ± 14.7 (114) |
| Fasting | 1.05 ± 0.08 (300)[c] | 79.7 ± 28.1 (71) | 144.6 ± 18.0 (128) |
| Diabetes | 0.84 ± 0.21 (240)[c] | 120.5 ± 19.5 (107) | 171.4 ± 16.9 (152) |
| Acetone | 1.60 ± 0.31 (457)[c] | 149.5 ± 21.7 (133) | 134.0 ± 6.1 (119) |

[a] nmol of *p*-aminophenol formed/min/mg of protein, at 37°.
[b] pmol of resorufin formed/min/mg of protein, at 25°.
[c] Significantly different from the control value ($p < 0.015$).

membranes were from DuPont-New England Nuclear (Boston, MA). Nitrocellulose membrane (pore size, 0.45 μm) was purchased from Schleicher and Schuell (Keene, NH). Other chemicals were the highest grade commercially available and other methods were described elsewhere (16, 19), if not specifically mentioned.

**Animal treatment.** Male outbred Sprague-Dawley rats (weighing 190–200 g) were obtained from Charles River Breeding Company (Raleigh, NC). Animals were group housed in polycarbonate cages on ground corncob bedding. Water was supplied in bottles with sipper tubes, and NIH 31 autoclavable rat and mouse diet was supplied *ad libitum*. The animal room environment was maintained at 21–23°, with humidity of approximately 50%, a ventilation rate of 15–20 air changes/hr of 100% outside air, and a 12-hr light-dark cycle. Throughout the study the animals were housed, maintained in accordance with National Institutes of Health guidelines, and treated for 3 days, unless specified otherwise.

One group of rats were treated with 3% acetone in drinking water (for 7 days), a second group with streptozotocin (60 mg/kg/day, for 3 days, intraperitoneally), and a third group with a home-made high fat diet (70% lard, 20% casein, 1.5% vitamin mixture, 3.5% salt mixture, and 5% methyl cellulose without carbohydrates), which markedly elevates the levels of ketone bodies. A fourth group was maintained without food for 48 hr, and the control group was given a normal diet. After treatment, animals were briefly anesthetized with diethyl ether, and whole blood was collected from cardiac puncture after an incision was made across the abdomen. The plasma was prepared for the analysis of ketone bodies, and major organs such as liver, lung, and kidney were immediately removed, frozen in liquid nitrogen, and stored at −80° until processed further.

**Preparation of microsomal fractions and enzyme assays.** Microsomal fractions of these tissues were prepared by differential centrifugation, as detailed previously (16, 19). The protein concentration was determined by the method of Lowry *et al.* (21), using bovine serum albumin as standard. Aniline hydroxylase activity was determined by measuring *p*-aminophenol formation as described (16). Pentoxyresorufin *O*-dependentylase and ethoxyresorufin *O*-deethylase activities were measured by the method of Lubet *et al.* (22).

**Immunoblot analyses.** The preparation and properties of polyclonal antibodies to P450IIE were described elsewhere (18). The goat polyclonal antibodies against P450IA (constitutively expressed P450 analogous to P450IA, inducible by polycyclic aromatic compounds) and P450IIC (constitutively expressed P450 analogous to P450IIB) were kindly provided by Dr. Frank J. Gonzalez (National Cancer Institute) or purchased from the Oxford Biomedical Research, Inc. (Oxford, MI). SDS-polyacrylamide gel electrophoresis, electrical transfer of the proteins, and subsequent Western immunoblots were performed as outlined (18). The nitrocellulose membranes were first incubated with 3% (w/v) nonfat dry milk dissolved in phosphate-buffered saline, pH 7.4, before incubation with primary specific antibodies. Electrophoretic bands were then recognized by the secondary antibodies, such as goat anti-rabbit IgG or rabbit anti-goat IgG conjugated with alkaline phosphatase (Kirkegaard and Perry Laboratories, Gaithersburg, MD), and were finally visualized by color development, using a mixture of 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium as the chromogenic substrate. The densities of immunoreactive and autoradiographic bands were measured by a laser computing densitometer (Molecular Dynamics, Sunnyvale, CA).

**Northern mRNA blot analysis.** Changes in P450IIE mRNA were analyzed by Northern blot analysis, using 0.66 M formaldehyde-1% agarose gels (23). Cytoplasmic RNA from rat livers from control and treated groups was prepared by the acid guanidinium thiocyanate-phenol-chloroform method (24). Transfer and blotting were performed using a GeneScreen membrane, as described (23). A near-full-length cDNA fragment (1.6-kb long) specific for rat P450IIE (16) was labeled by nick translation, using a kit supplied (Bethesda Research Laboratories, Gaithersburg, MD) or a random-primed labeling kit and [$\alpha$-$^{32}$P] dCTP (specific activity, 3000 Ci/mmol; Amersham Corp., Arlington Heights, IL). In separate experiments (data not shown), all RNAs on

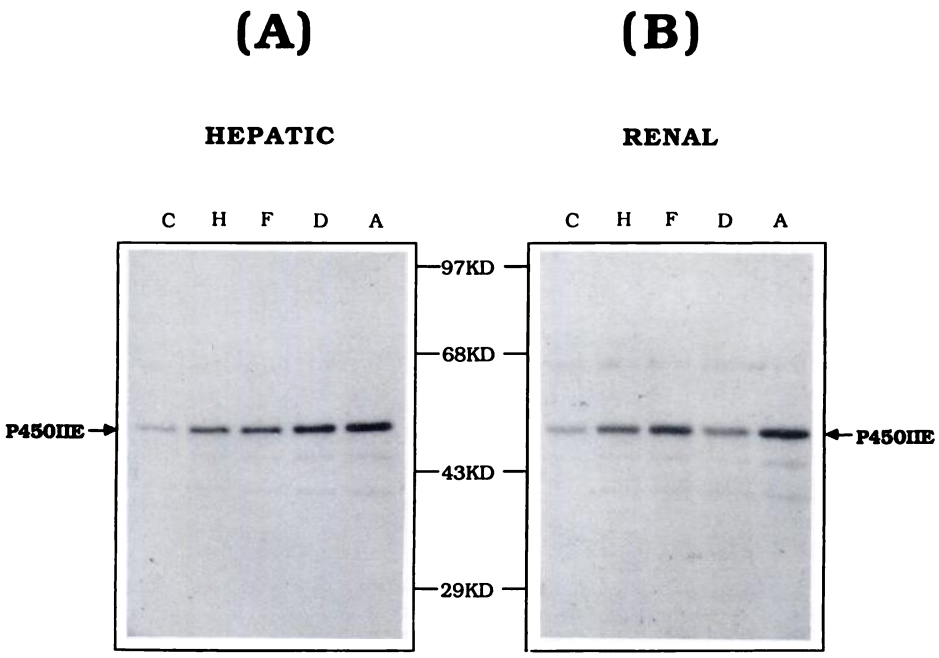


**Fig. 1.** Immunoblot analyses for P450IIE in hepatic (A) and renal (B) microsomes. Microsomal proteins from control and treated rats (5 μg/well for hepatic and 50 μg/well for renal microsomes) were separated on 10% SDS-polyacrylamide gels, transferred to nitrocellulose filters, and then subjected to immunoblot analyses with polyclonal antibodies against P450IIE, as detailed in the Materials and Methods. Microsomes were prepared from untreated control rats (*C*) and rats fed a high fat diet (*H*) or acetone (*A*). Fasted rats (*F*) and streptozotocin-induced diabetic rats (*D*) were also included. The molecular size markers were phosphorylase *b* (97 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), and carbonic anhydrase (29 kDa).

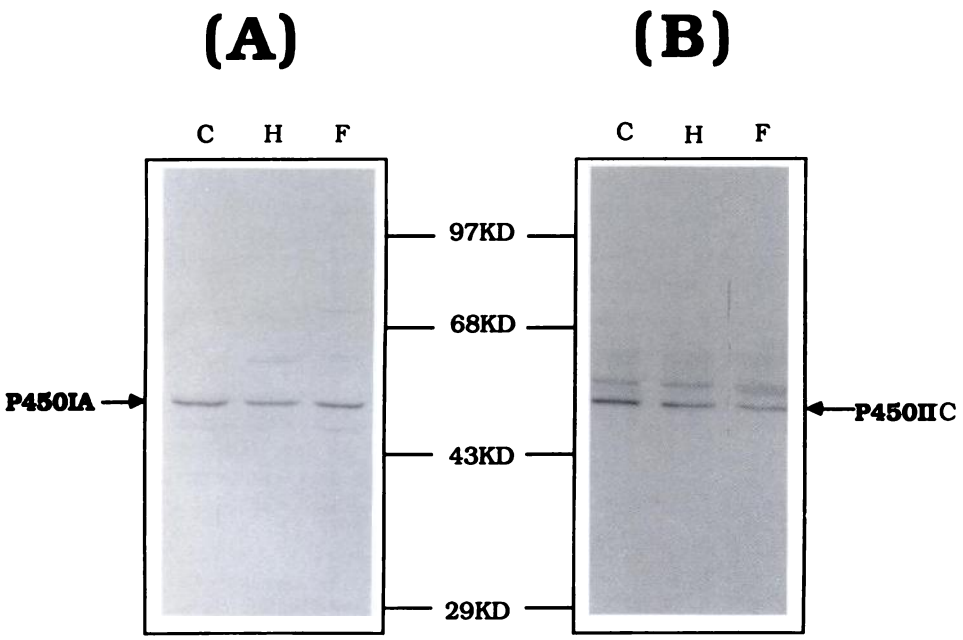


**Fig. 2.** Immunoblot analyses for P450IA (A) and P450IIC (B). Hepatic microsomal proteins (5 μg/well) were separated on 10% SDS-polyacrylamide gels and subjected to immunoblot analyses with polyclonal antibodies against P450IA (A) and P450IIC (B). The experimental conditions were the same as described in Fig. 1 legend.

duplicate blots were also analyzed for the levels of actin mRNA, probed with $^{32}$P-labeled DNA probe (1.15-kb long, *Pst*I-digested fragment) specific for mouse β-actin gene (kindly provided by Dr. Shioko Kimura at National Cancer Institute), as an internal control. Prehybridization and hybridization were performed by the method of Virca *et al.* (25), with a slight modification. The hybridization was carried out at 63° in a solution of 50 mM PIPES, 100 μg/ml denatured salmon sperm DNA, 5× Denhardt's solution (1× Denhardt's contains 0.02% each of Ficoll, polyvinylpyrrolidone, and bovine serum albumin), 100 mM NaCl, 50 mM sodium phosphate, 1 mM EDTA, and 5% SDS. After hybridization with $^{32}$P-labeled cDNA probes for P450IIE and actin, the membranes were washed at room temperature for 10 min with 1× standard saline citrate (1× standard saline citrate is 0.15 M sodium chloride and 0.015 M sodium citrate, pH 7.0), with 5% SDS. After this wash, blots were washed twice for 20 min with the latter solution as described above, at 63°. Washed membranes were exposed for varying lengths of time, at −80°, to X-ray films (Eastman Kodak Company, Rochester, NY), with intensifying screens.

**Analyses of plasma ketone bodies.** Serum acetone was measured by gas chromatography, following the method of Gavino *et al.* (26), with slight modification. Before injection onto a 80/120 Carbopak B/5% Carbowax 20M column (Alltech, Deerfield, IL), serum was made 0.1 mM in *n*-propanol and spun through a 10,000-Da cut-off spin column (Millipore) for 30 min at 3°, in an Eppendorf centrifuge. This decreased sample build-up on the column head. Serum acetoacetate and β-hydroxybutyrate were measured enzymatically (27, 28).

## Results

**Changes in animal weight during treatment.** In order to verify our results with a high fat diet, the animals were pair-fed and monitored for their food consumption and weight gains during the period of treatment. All fed experimental groups gained the same amount of body weight (about 25 g; $p < 0.01$), whereas fasted rats lost weight (about 28 g; $p < 0.01$). Based on normal food consumption and weight gains, the results of fat feeding appeared to be due to direct effects of fat or its metabolites on P450IIE induction.

**Induction of P450IIE enzyme activity and immunoreactive protein content.** In agreement with earlier studies (5, 6, 16), aniline hydroxylase activity was increased 3–4-fold in the acetone-treated, fasting, and diabetic rats. P450IIE activity was also increased significantly ($p < 0.015$) in rats fed a high fat diet (Table 1). However, the catalytic activities of pentoxyresorufin *O*-dependentylase and ethoxyresorufin *O*-deethylase associated with P450IIC and P450IA, respectively, did not change during the aforementioned treatments (Table 1). To investigate whether the enhanced P450IIE-mediated enzyme activities under these conditions were accompanied by increases of P450IIE protein in hepatic and renal microsomes, immunoblot analyses after SDS-polyacrylamide gel electrophoresis were performed. As shown in Fig. 1, the band intensities of immunoreactive P450IIE for treated groups were higher than those for controls in both hepatic and renal microsomes. The densitometric analysis of the immunoblots showed that the levels of P450IIE protein in rats with various treatments increased 3–4-fold over the control. When polyclonal anti-P450IA (Fig. 2A) and anti-P450IIC (Fig. 2B) antibodies were used in the immunoblot analysis, no differences in the amounts of immunoreactive P450IA and P450IIC were observed between fat-treated, fasted, and control groups. These results suggest that P450IIE can be specifically and strongly induced during ketosis caused by either fat feeding or fasting (Fig. 1).

**Changes in P450IIE mRNA level.** To further explore the mechanism of P450IIE induction after a fat diet, the level of P450IIE mRNA was examined by estimating the ratio of P450IIE mRNA to total RNA (or actin mRNA used as an internal standard). Consistent with our previous results on control and induced rats (16, 18), Northern blot analysis revealed that higher levels of P450IIE mRNA were observed in fasting and diabetic rats, with no differences in acetone-treated rats (data not shown). The changes in P450IIE mRNA after a fat feeding were also studied by separate Northern blot analyses. The densitometric analysis of the ribosomal subunits (Fig. 3A) showed that comparable amounts of total RNA were applied in each well, except for the last three lanes (from the right), where about 30% more RNA was used. Consistent with our earlier results (16, 18), a single species of P450IIE mRNA (about 1.8-kb long) was observed (Fig. 3B) with the specific

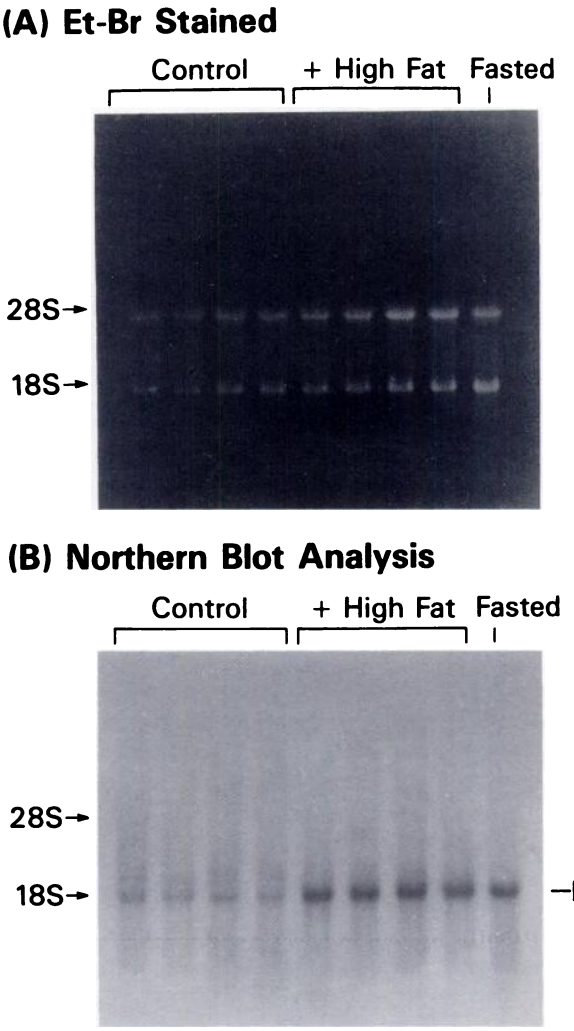


**Fig. 3.** Northern blot analysis for the levels of P450IIE mRNA. Total cellular RNAs (15 μg/well) were separated on agarose gels and subjected to Northern blot analysis, using a specific cDNA probe for P450IIE (B), as described in Materials and Methods. A, Ethidium bromide staining pattern of the gel containing 28 S and 18 S rRNA subunits.

TABLE 2

**Levels of plasma ketone bodies in rats after various treatments**

The values represent mean ± standard error (four experiments for each group, repeated twice).

| Treatment | Acetone | β-Hydroxybutyrate | Acetoacetate |
|---|---|---|---|
| | μM | mM | mM |
| Control | 6.77 ± 1.59 | 0.16 ± 0.02 | 0.03 ± 0.008 |
| High fat | 22.80 ± 3.20 | 1.52 ± 0.19 | 0.60 ± 0.12 |
| Fasting | 48.52 ± 4.54 | 3.20 ± 0.34 | 0.54 ± 0.10 |
| Diabetes | 14.65 ± 5.31 | 0.88 ± 0.42 | 0.22 ± 0.10 |
| Acetone | 7774 ± 410 | 0.10 ± 0.008 | 0.04 ± 0.009 |

cDNA probe. In addition, densitometric analysis of the Northern blot revealed that the levels of P450IIE mRNA in high fat-fed and fasted (as a positive control) rats were increased about 3-fold over the control. However, the levels of actin mRNA for the same groups did not significantly differ from the control ($p < 0.05$). These data thus indicate that P450IIE mRNA was specifically elevated by a high fat feeding and that the degree of P450IIE mRNA elevation generally agreed with the increases in P450IIE protein and its catalytic activity.

**Plasma levels of ketone bodies and correlation with P450IIE mRNA level.** In order to delineate the induction mechanism observed in the treated animals, the levels of plasma ketone bodies were determined and summarized (Table 2). In the fasted, diabetic, and high fat diet-treated rats, acetoacetate and β-hydroxybutyrate levels were markedly elevated (>10-fold in fasted and fat-treated rats) over the control group. However, the difference in acetone level between control and treated groups was not as great as that in the acetone-treated rats, where acetone level was about 1000-fold higher than control (Table 2). The densitometric levels of P450IIE mRNA for the rats whose ketone body levels were determined correlated positively ($r = 0.73$) with the plasma concentrations of acetoacetate and β-hydroxybutyrate (Fig. 4) but not with that of acetone, which did not elevate the P450IIE mRNA level. The present data agree with earlier data (18, 19), where acetone appears to increase P450IIE activity and immunoreactive protein via a posttranslational mechanism (P450IIE protein stabilization).

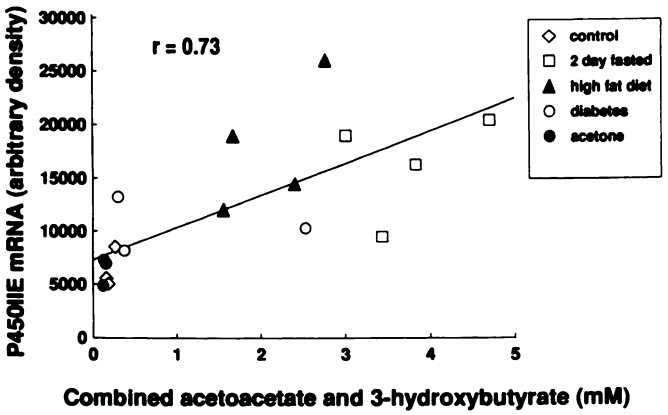


**Fig. 4.** Correlation between the levels of two ketone bodies and P450IIE mRNA. The levels of P450IIE mRNA for all the animals were densitometrically determined. The density of P450IIE mRNA and the sum of the concentrations of the two ketone bodies were plotted, and the correlation coefficient was calculated by the least squares regression method.

## Discussion

Unlike the regulation of most of the other major classes of P450s, which are induced via transcriptional activation by their respective inducers (1, 3), the regulation of P450IIE expression is more complex. P450IIE appears to be activated at every step of protein synthesis, i.e., transcriptional activation during normal development, probably due to demethylation of cytosine residues in the 5′ flanking region of the rat P450IIE gene (16, 17), pretranslational activation with an increase in P450IIE mRNA observed in diabetes (18), fasting (29, 30), and a high fat diet (this study), and posttranslational activation, without elevation of P450IIE mRNA, by its own substrates, such as acetone, ethanol, and pyridine (18, 19, 31–33). In addition, its expression can be down-regulated by circulating growth hormone (34, 35) or the suicide substrate $CCl_4$ (36). Based on the multiple types of regulation of P450IIE expression described above, the regulation of P450IIE expression represents an unique example of protein regulation.

In contrast to the earlier study (14) with exogenous ketone bodies, an endogenous accumulation of ketone bodies was achieved with a high fat diet, where P450IIE was specifically induced (Figs. 1 and 2; Table 1). The P450IIE elevation, however, was accompanied (probably preceded) by a 3-fold increase in its mRNA level, suggesting a pretranslational activation

mechanism. The P450IIE induction in liver and kidney (Fig. 1) suggests that the steady state level of P450IIE mRNA in fat-fed rats could be regulated, directly or indirectly, by unknown factor(s) present in plasma, whose level(s) may also fluctuate in the ketotic states. Growth hormone could be such a candidate, since Yamazoe *et al.* (35, 39) suggested that the induction of P450IIC, P450IA, and P450IIE in diabetic animals could be due to a decrease in the level of growth hormone, which suppresses the expression of constitutively expressed P450s, including P450IIE. Although we did not measure the level of growth hormone in the current study, our results on specific induction of P450IIE without changes in P450IA and P450IIC (Table 1; Fig. 2) indicated that growth hormone may not play a significant role in P450IIE induction during ketosis caused by fat feeding. Our hypothesis is also consistent with the recent data on the lack of a role of growth hormone in P450IIE induction observed in diabetic rats (20, 40).

Alternatively, P450IIE in ketotic conditions could be induced by another common factor whose level would be elevated in parallel with the increase in P450IIE mRNA level. Diabetic ketoacidosis and prolonged starvation are characterized by similar metabolic changes, i.e., decreased glucose utilization, increased hepatic gluconeogenesis, increased fatty acid catabolism, and increased levels of ketone bodies. All three ketone bodies, acetone, $\beta$-hydroxybutyrate, and acetoacetate, are obvious candidates. These metabolites increase with starvation, and all three play a role in limited conversion of acetone to glucose, as observed in humans and rats during periods of starvation and diabetic ketoacidosis (37, 38), where P450IIE could participate in gluconeogenesis via acetone metabolism, as proposed (6, 12). The same metabolites that are increased in ketotic conditions (Table 2) could also explain the increase in P450IIE mRNA observed in high fat feeding. However, the data presented in Fig. 3 and Table 2 clearly show that serum acetone has no effect on rat P450IIE mRNA. On the other hand, serum acetoacetate and $\beta$-hydroxybutyrate were elevated by fat feeding to levels similar to those observed during starvation and diabetes and were positively correlated with P450IIE mRNA level (Fig. 4), although we could not establish a direct relationship between the two parameters; this warrants further study. Our preliminary data (not shown) from a nuclear run-off transcription assay for both control and fat-fed rats did not show significant differences in the rates of transcription for P450IIE and actin. Although we do not know what actually causes the elevation of P450IIE mRNA in ketotic states, the data presented here suggest that the P450IIE induction by a high fat feeding appears be due to pretranslational activation accompanied by an increase in P450IIE mRNA, similar to that observed in fasted (29) and diabetic animals (18, 30).

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