The hepatic CYP4A enzymes are important fatty acid and prostaglandin ω-hydroxylases that are highly inducible by fibric acid hypolipidemic agents and other peroxisome proliferators. Induction of the CYP4A enzymes by peroxisome proliferators is mediated through the nuclear peroxisome proliferator-activated receptor α (PPARα). Fatty acids have recently been identified as endogenous ligands of PPARα, and this receptor has been implicated in the regulation of lipid homeostasis. In the present report we characterized the induction of the hepatic CYP4A genes in rats during the altered lipid metabolism associated with starvation and diabetes. The mRNA levels of CYP4A1, CYP4A2, and CYP4A3 were induced 7–17-fold in the livers of fasted animals and 3–8-fold in the livers of diabetic animals. This was accompanied by corresponding changes in CYP4A protein levels and arachidonic and laurie acid ω-hydroxylase activity. Interestingly, feeding animals after the fasting period caused as much as an 80% suppression of CYP4A mRNA levels, whereas CYP4A protein levels and functional activity returned to control values. A second PPARα-responsive gene, acyl-CoA oxidase, was also induced in rat liver by diabetes and fasting. By using PPARα-deficient mice, we unambiguously demonstrated that PPARα is strictly required for hepatic CYP4A induction by starvation and diabetes. Similarly, induction of hepatic thiolase and bifunctional enzyme also required expression of PPARα. This represents the first evidence for the pathophysiologically induced activation of a nuclear receptor.

The cytochrome P450 4A (CYP4A) enzymes are fatty acid and prostaglandin ω-hydroxylases that are abundantly expressed in the liver and kidney (1). Of recent interest has been the physiological significance of CYP4A-catalyzed arachidonic acid metabolites. The ω-hydroxylated metabolite of arachidonic acid, 20-hydroxyeicosatetraenoic acid (20-HETE), has been implicated in diverse biological functions, including the regulation of vascular tone, renal tubular ion transport, and bronchoconstriction (2). Recent studies with recombinant proteins have confirmed the narrow substrate specificity of the CYP4A enzymes. These enzymes can hydroxylate saturated and unsaturated fatty acids at the ω and ω-1 positions, with a clear preference for the ω position (3, 4). The selectivity of the CYP4A enzymes for prostaglandins E₁, A₁, and F₂α is limited to specific isoforms, and hydroxylation of these substrates is measurable only at the ω position (3, 4). Detailed kinetic studies have not yet been carried out to clearly identify the relative importance of the multiple CYP4A isoforms in the in vivo metabolism of fatty acids and prostaglandins. Fatty acid ω-hydroxylation is generally a minor pathway of hepatic fatty acid metabolism relative to mitochondrial β-oxidation. However, it becomes increasingly important during periods of increased delivery of fatty acids to the liver such as in uncontrolled diabetes mellitus or starvation and in clinical conditions such as Reye’s syndrome and alcoholic liver disease where mitochondrial β-oxidation is severely impaired (5–7).

The hepatic and renal expression of the CYP4A genes is highly inducible by a diverse group of compounds referred to as peroxisome proliferators. Peroxisome proliferators include the widely prescribed lipid-lowering drugs of the fibrate class (including clofibrate, fenofibrate, and nafenopin), phthalate ester plasticizers such as di-(2-ethylhexyl)phthalate, the endogenous steroid dehydroepiandrosterone, and chlorinated phenoxy and benzoic acid herbicides (8–12). Administration of peroxisome proliferators to rats results in hepatomegaly, a dramatic increase in the number of peroxisomes in the liver, and a decreased level of serum triglycerides (13). An induction of a number of genes that encode proteins involved in fatty acid metabolism and transport accompanies these changes in liver structure and lipid metabolism. Included in this group are the CYP4A enzymes, acyl-coenzyme A oxidase (AOX), the bifunctional enzyme (BIEN), 3-ketoacyl-CoA thiolase (thiolase), the cytosolic liver fatty acid-binding protein, HMG-CoA synthase, and apolipoproteins A-I and A-II (14).

significant progress has been made in understanding the mechanisms involved in the induction of CYP4A and other responsive genes by peroxisome proliferators. Early studies indicated a transcriptional regulation of induction characterized by a 5–7-fold increase in CYP4A mRNA levels following treatment with clofibrate (15). Induction is regulated through the peroxisome proliferator-activated receptor (PPAR), a member of the nuclear receptor superfamily of ligand-activated transcription factors. Three different PPARs have been identified (α, β/δ, and γ), and each displays a distinct pattern of expression (14). The α isoform is predominantly expressed in
hepatocytes, cardiomyocytes, renal proximal tubule cells, and enterocytes and is associated with the peroxisome proliferator effects. Targeted disruption of PPARα in mice clearly demonstrated that this was the major isoform mediating the pleiotropic effects of peroxisome proliferators (16). Following treatment with the peroxisome proliferators clofibrate and Wy-14,463, mice lacking the PPARα gene showed no hepatomegaly, peroxisome proliferation, or induction of the normally responsive genes. PPARs contain a DNA-binding domain that recognizes a peroxisome proliferator response element in the promoters of target genes. Peroxisome proliferator response elements consist of a directly repeating core sequence separated by one nucleotide and have been identified in a number of genes involved in lipid metabolism and transport, including rabbit CYP4A6 and rat CYP4A1 (17). The PPAR binds to the response element as a heterodimeric complex with the 9-cis-retinoic acid (rexinoid) receptor (18). PPARα was originally considered an orphan receptor with no identified endogenous ligand. In addition to peroxisome proliferators, a number of fatty acids have been shown to activate PPARs, but until recently, attempts to demonstrate binding of these compounds to the receptor failed (19–21). In the past year several groups have identified unsaturated fatty acids, the lipoxigenase metabolite (8S)-HETE, and fibrates as ligands for PPARα (22, 23). Based on the results of competition binding and conformation-based assays, oleic, linoleic, linolenic, and arachidonic acids were identified as ligands at concentrations similar to those found in human plasma, providing strong evidence that these compounds act as endogenous ligands of PPARα. Other eicosanoids activate but do not bind the receptor, raising the possibility that further metabolism may be required for the formation of the actual ligand. Recent data show that in rodents dietary polysaturated fatty acids induce microsomal and peroxisomal fatty acid oxidation through activation of PPARα and suppress lipogenic gene expression through a PPARα-independent mechanism (24). The identification of fatty acids and eicosanoids as PPAR ligands suggests that this receptor plays an important role in the regulation of lipid homeostasis and in the pathogenesis of a number of metabolic disorders, including diabetes, obesity, and atherosclerosis.

Limited data suggest that the hepatic CYP4A genes are also induced by at least two pathophysiological conditions, diabetes and fasting. An increase in CYP4A protein levels and its specific enzymatic activity, lauric acid (ω-1)-hydroxylase activity, has been detected in the livers of streptozotocin-treated rats (25–28). Limited data suggest that CYP4A mRNA levels are also increased following induction of diabetes (28, 29). Similarly, CYP4A protein levels and associated lauric acid hydroxylation were increased in hepatic microsomes from animals that had been fasted for 48–72 h immediately prior to sacrifice (30). There is no information from these studies as to whether CYP4A mRNA levels were also increased.

The current study was designed to test the hypothesis of involvement of the nuclear receptor PPARα in the pathophysiological adaptive response of mammals to diabetes and fasting. This is of particular importance since induction of the formation of the CYP4A metabolites of arachidonic acid may alter their biological effects. We found that both diabetes and fasting induce CYP4A mRNA and protein expression and alter the profile of arachidonic acid metabolism. The diabetes-induced effects were insulin-dependent, and the effects due to fasting were highly dependent on the duration of food deprivation. By using PPARα-deficient mice (PPARα−/−), we unambiguously demonstrated that these effects are mediated through PPARα, providing the first evidence of a pathophysiologically induced activation of this nuclear receptor. Furthermore, similar induction of AOX, BIEN, and thiolase suggests that this pathophysiologically activation of PPARα will have widespread effects on hepatic lipid metabolism.

### EXPERIMENTAL PROCEDURES

#### Materials

Streptozotocin was purchased from Sigma and insulin from the University of California San Francisco Pharmacy Services. Blood glucose was measured using the One Touch® monitoring kit from Lifescan, Milpitas, CA. Radiolabeled nucleotides were purchased from NEN Life Science Products, and radiolabeled arachidonic acid and lauric acid were from Amersham Pharmacia Biotech. Restriction enzymes were obtained from New England Biolabs, Beverly, MA; modifying enzymes were from Life Technologies, Inc., and RNase was from Fisher. Arachidonic acid and lauric acid were purchased from Nu Chek Prep, Elysian, MN. Nitrocellulose and nylon membranes were from Micron Separations, Inc., Westborough, MA, and the anti-rat CYP4A1 antiserum was purchased from Gentest Corp., Woburn, MA. Protein was measured with the Pierce BCA Protein Assay from Pierce. All other reagents were of the highest grade available and were purchased from Fisher or Sigma.

#### Animal Treatment

Male Sprague-Dawley rats (200–220 g) were purchased from Simonsen Laboratories, Gilroy, CA, and were housed in a controlled environment with a 12-h light/dark cycle and free access to water and standard laboratory chow. All animal use was approved by the University of California at San Francisco Committee on Animal Research and followed the National Institutes of Health guidelines for the care and use of experimental animals. Diabetes was induced by a single intraperitoneal injection of streptozotocin (65 mg/kg) in 50 ml citrate buffer, pH 4.5. Control animals were given an injection of buffer only. One group of diabetic animals was given subcutaneous injections of insulin (2 units at 8:00 a.m. and 4 units at 8:00 p.m.) on days 14–20 following streptozotocin treatment. Body weight and blood glucose were determined prior to induction of diabetes and weekly throughout the study period. All animals were sacrificed 21 days after streptozotocin injection. In the fasting studies, food was removed from the animals for 48 h and the animals were sacrificed at the end of the fasting period. In some cases, the fasting period was followed by a period in which animals were fed for various lengths of time before sacrifice. Control animals had free access to food for an identical length of time, and all groups of animals had free access to water throughout the study. In all cases, rats were anesthetized with ether, and their livers were perfused with ice-cold saline, removed, and immediately frozen in liquid nitrogen. The livers were stored at −80 °C for the preparation of RNA and microsomes.

Similar diabetes and fasting protocols were also carried out in C57BL/6 control mice and PPARα−/− mice. All of the mouse studies were carried out at Laboratoire de Pharmacologie et Toxicologie in Toulouse, France, according to institutional guidelines. The development of the PPARα−/− mice line from homologous recombination of 129Sv-derived cells has been described previously (16). Chimeric males were initially backcrossed to C57BL/6 females. Several additional rounds of backcrossing were performed in our animal facility to increase the C57BL/6 genetic background and to generate the animals used in these studies. C57BL/6 controls were from IFFA-CREDO, Les Oncins, France. Diabetes was induced in control and PPARα−/− mice (5 mice/strain) with a single intraperitoneal injection of 170 mg/kg streptozotocin. Control animals received vehicle only. Ten days after the streptozotocin treatment the mice were sacrificed, and blood was collected for blood glucose determinations, and livers were removed and immediately frozen in liquid nitrogen. Six groups of five mice were used for the fasting protocol. Control groups of C57BL/6 and PPARα−/− mice were allowed free access to food, and the remaining two groups of each strain were fasted for 30 h. One set of fasted animals was sacrificed at the end of this period, and a second set was fed for 42 h before sacrifice. All animals had free access to water throughout the entire experimental period. At the end of the experimental period, blood was collected for glucose determinations, and livers were removed and immediately frozen in liquid nitrogen.

#### Ribonuclease Protection Assays

Total RNA was isolated from livers by acid/phenol extraction (31). Construction of specific CYP4A riboprobes and details of the ribonuclease protection assays were described previously (32). The CYP4A3 probe is used to detect both the CYP4A3 and CYP4A2 mRNA transcripts. The protected fragments spanned the following regions of the cDNA sequence: 1285–1557 nt of CYP4A1; 343–549 nt of CYP4A2; 214–526 nt of CYP4A3; and 2–250 nt of GAPDH. Autoradiographs were scanned with a Ultro Scan XL laser scanner.
PPARα Activation by Starvation and Diabetes

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Effect of diabetes on hepatic lauric acid and arachidonic acid metabolism

Metabolite formation rates were measured in duplicate from microsomes prepared from six animals per treatment group. Values shown are the mean ± S.D. for each treatment group. Groups of six rats were injected on day 1 with a single dose of streptozotocin (diabetic and insulin groups) or vehicle. The insulin-treated group was given insulin twice daily between days 14 and 21, and all animals were sacrificed on day 21. Hepatic microsomes were prepared, and metabolism of lauric acid (100 μM) and arachidonic acid (250 μM) was measured as described under “Experimental Procedures.” Epoxidease activity is the sum of epoxyeicosatrienoic acid and dihydroxyeicosatrienoic acid formation. Treatment groups were compared using an analysis of variance and post hoc multiple comparisons with a modified t test.

### TABLE I

| Metabolite formation rate | Control | Diabetic | Insulin |
|--------------------------|---------|----------|---------|
| 11-OH lauric acid        | 0.012   | 0.013    | 0.012   |
| 12-OH lauric acid        | 0.012   | 0.013    | 0.012   |
| 19-HETE                  | 0.012   | 0.013    | 0.012   |
| 20-HETE                  | 0.012   | 0.013    | 0.012   |
| Epoxygenase              | 0.012   | 0.013    | 0.012   |

### RESULTS

Induction of CYP4A with Diabetes—All rats treated with streptozotocin showed a significant decrease in weight gain and increase in blood glucose levels relative to controls, consistent with the induction of diabetes. One week following streptozotocin treatment blood glucose levels were 88 ± 11 mg/dl in control animals and 379 ± 64 mg/dl in the diabetic animals (p < 0.005). This elevation in blood glucose was maintained throughout the entire study period in the untreated diabetic rats. Both weight gain and blood glucose levels returned to control values upon treatment of diabetic rats with insulin. The effect of diabetes on CYP4A mRNA levels was measured using gene-specific RNase protection assays. These assays quantify the message level of each individual CYP4A gene in a given sample relative to the level of GAPDH mRNA. Diabetic animals showed a significant induction of CYP4A mRNA levels (Fig. 1). The message level for each of the hepatically expressed CYP4A genes (CYP4A1, CYP4A2 and CYP4A3) was increased up to 8-fold in the diabetic rat livers, and this increased expression was completely reversed by treatment of diabetic rats with insulin. The order of induction was CYP4A1 > CYP4A2 > CYP4A3.

Lauric acid and arachidonic acid ω- and (ω-1)-hydroxylase activity were measured in liver microsomes as indicators of CYP4A functional activity. Hydroxylation at the terminal position of both of these fatty acids is catalyzed solely by the CYP4A enzymes, whereas metabolism at the ω-1 position is also dependent on CYP2E1 and CYP2C enzymes (3, 34–36). Lauric acid was metabolized at the ω and ω-1 position in roughly a 1:1 ratio. Lauric acid ω- and (ω-1)-hydroxylation were increased almost 2-fold in hepatic microsomes prepared from diabetic rats, and this activity returned to control values after treatment of diabetic animals with insulin (Table I). Arachidonic acid was metabolized at the ω and ω-1 positions in a 1:8.1

Fig. 1. Induction of hepatic CYP4A mRNA levels during diabetes. Groups of six rats were injected on day 1 with a single dose of streptozotocin (diabetic and insulin groups) or vehicle. The insulin-treated group was given insulin twice daily between days 14 and 21, and all animals were sacrificed on day 21. Total hepatic RNA was prepared and used for RNase protection assays. The autoradiograms are representative of six samples per treatment group. RNA was hybridized with a CYP4A1 (A) or a CYP4A3 (B) probe. The CYP4A3 probe was used to detect both CYP4A2 and CYP4A3. A rat GAPDH probe was included in all hybridizations. Autoradiograms were scanned with a laser densitometer, and the level of a given CYP4A mRNA in each sample was expressed relative to the level of GAPDH. The mean value ± S.D. of the CYP4A mRNA level from duplicate determinations of six samples/group is shown in C. *, p < 0.001 relative to control. Each of the CYP4A genes were significantly induced in the diabetic livers.

Hepatic Fatty Acid Metabolism—Hepatic microsomes were prepared from frozen liver tissue using differential centrifugation and stored at −80 °C (33). Arachidonic acid and lauric acid metabolism were measured in hepatic microsomes at a final substrate concentration of 0.25 and 0.10 mM, respectively. Reaction conditions, metabolite extraction, and separation and quantification of arachidonic acid and lauric acid metabolites by reverse phase high pressure liquid chromatography with radiometric detection were described previously (32).

Immunoblotting of CYP4A Proteins—Hepatic microsomes (5 μg of protein) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by Western immunoblotting using goat polyclonal antibodies against rat liver CYP4A1 (31). The electrophoresis conditions, protein transfer, and immunodetection have been described previously in detail (32).

Northern Blotting of CYP4A, AOX, BIEN, and Thiolase—Hepatic message levels of mouse CYP4A, BIEN, and thiolase and rat AOX were detected by Northern blotting. Total hepatic RNA was isolated with Trizol reagent from Life Technologies, Inc., Paris, France, and 10-μg samples were fractionated on a 1% agarose gel containing 2.2 m formaldehyde and transferred to a nylon membrane. A 1.8-kb fragment of the CYP4A3 cDNA, a 0.5-kb fragment of rat AOX cDNA, a 1.1-kb fragment of rat BIEN cDNA, a 1.0-kb fragment of rat thiolase cDNA, and a 1.2-kb fragment of rat GAPDH cDNA were labeled with [α-32P]dCTP using the random primer technique (31). The membranes were hybridized with the radiolabeled probe at 65 °C overnight in a solution of 6× SSC (1× SSC: 150 mM NaCl, 15 mM sodium citrate, pH 7.0), 1× Denhardt’s reagent (10 mg/ml each of BSA fraction V, Ficoll, and polyvinylpyrrolidone), 0.5% sodium dodecyl sulfate, and 20 μg/ml salmon sperm DNA. After hybridization the blots were washed to a final stringency of 0.1× SSC, 0.1% SDS at 65 °C. Bands were visualized with a PhosphorImager and analyzed using ImageQuant Software, Molecular Dynamics, Sunnyvale, CA.

Statistics—All measurements were performed on RNA or protein samples from individual animals, and the results are expressed as mean ± S.D. of five to six animals per treatment group. Statistical significance of differences between treatment groups was evaluated by an analysis of variance with post hoc multiple comparison testing with a modified t test. A value of p < 0.05 was considered to be statistically significant.

Control Diabetic Insulin

| Metabolite formation rate | Control | Diabetic | Insulin |
|--------------------------|---------|----------|---------|
| 11-OH lauric acid        | 0.012   | 0.013    | 0.012   |
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| Epoxygenase              | 0.012   | 0.013    | 0.012   |

### STRINGENCY

- 0.1× SSC, 0.1% SDS at 65 °C
- Bands were visualized with a PhosphorImager and analyzed using ImageQuant Software, Molecular Dynamics, Sunnyvale, CA.

- All measurements were performed on RNA or protein samples from individual animals, and the results are expressed as mean ± S.D. of five to six animals per treatment group.

- Statistical significance of differences between treatment groups was evaluated by an analysis of variance with post hoc multiple comparison testing with a modified t test. A value of p < 0.05 was considered to be statistically significant.

### RESULTS

- Induction of CYP4A with Diabetes—All rats treated with streptozotocin showed a significant decrease in weight gain and increase in blood glucose levels relative to controls, consistent with the induction of diabetes.
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- This elevation in blood glucose was maintained throughout the entire study period in the untreated diabetic rats.
- Both weight gain and blood glucose levels returned to control values upon treatment of diabetic rats with insulin.
- The effect of diabetes on CYP4A mRNA levels was measured using gene-specific RNase protection assays. These assays quantify the message level of each individual CYP4A gene in a given sample relative to the level of GAPDH mRNA.
- Diabetic animals showed a significant induction of CYP4A mRNA levels (Fig. 1).
- The message level for each of the hepatically expressed CYP4A genes (CYP4A1, CYP4A2 and CYP4A3) was increased up to 8-fold in the diabetic rat livers, and this increased expression was completely reversed by treatment of diabetic rats with insulin.
- The order of induction was CYP4A1 > CYP4A2 > CYP4A3.

- Lauric acid and arachidonic acid ω- and (ω-1)-hydroxylase activity were measured in liver microsomes as indicators of CYP4A functional activity.
- Hydroxylation at the terminal position of both of these fatty acids is catalyzed solely by the CYP4A enzymes, whereas metabolism at the ω-1 position is also dependent on CYP2E1 and CYP2C enzymes (3, 34–36).
- Lauric acid was metabolized at the ω and ω-1 position in roughly a 1:1 ratio.
- Lauric acid ω- and (ω-1)-hydroxylation were increased almost 2-fold in hepatic microsomes prepared from diabetic rats, and this activity returned to control values after treatment of diabetic animals with insulin (Table I).
- Arachidonic acid was metabolized at the ω and ω-1 positions in a 1:8.1
CYP4A genes in a time-dependent fashion.

6 described for Fig. 1. The mean value for RNase protection assays with a CYP4A1 (A) probe as described for Fig. 1. Total hepatic RNA was prepared and used for RNase protection assays with a CYP4A1 (A) or CYP4A3 (B) probe as described for Fig. 1. The mean value ± S.D. of the CYP4A mRNA level from duplicate determinations of six samples/group is shown in C. *, p < 0.005; †, p < 0.0001 relative to control. Fasting induced each of the CYP4A genes in a time-dependent fashion. Induction of both CYP4A mRNA and protein levels were significantly suppressed in all treatment groups.

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Metabolite formation rates were measured in duplicate from microsomes prepared from six animals per treatment group. Livers were harvested from fasted animals after the indicated period. Fed animals were fasted for the indicated period and then allowed free access to food for the identical length of time before harvesting livers. Hepatic microsomes were prepared, and metabolism of lauric acid (100 μM) and arachidonic acid (250 μM) was measured as described under "Experimental Procedures." Epoxide activity is the sum of epoxyeicosatrienoic acid and dihydroxyeicosatrienoic acid formation. Treatment groups were compared using an analysis of variance and post hoc multiple comparisons with a modified t test.

| Metabolite formation rate | Fasting | Fed |
|--------------------------|---------|-----|
|                         | Control | 24 h | 48 h | 72 h | Control | 24 h | 48 h | 72 h |
| 11-OH lauric acid        | 775 ± 160 | 941 ± 190 | 1953 ± 382<sup>c</sup> | 1599 ± 687<sup>b</sup> |
| 12-OH lauric acid        | 564 ± 106 | 789 ± 55  | 2140 ± 386<sup>b</sup> | 1423 ± 552<sup>b</sup> |
| 19-HETE                  | 97.0 ± 18.0 | 60.4 ± 15.1<sup>c</sup> | 141 ± 35.1<sup>c</sup> | 98.5 ± 32.1<sup>c</sup> |
| 20-HETE                  | 185 ± 37.2 | 136 ± 10.8 | 279 ± 68.3<sup>c</sup> | 173 ± 36.9<sup>c</sup> |
| Epoxide                  | 1571 ± 393 | 760 ± 311<sup>c</sup> | 2080 ± 780 | 936 ± 300<sup>c</sup> |

<sup>a</sup> p < 0.0005.  <sup>b</sup> p < 0.005.  <sup>c</sup> p < 0.05.

Arachidonic acid ω- and (ω-1)-hydroxylations were increased only in the 48-h fasted animals, and the absolute increase in this activity was significantly less than that measured for lauric acid. For example, after a 48-h fast ω-hydroxylase activity increased 3.8-fold for lauric acid and only 1.5-fold for arachidonic acid. Induction of arachidonic acid epoxide activity also was only apparent with the 48-h fast and was minimal (1.3-fold). Surprisingly, all pathways of arachidonic acid metabolism were suppressed to some degree following the 24-h fasting period. Arachidonic acid was, however, a better marker for the decreased CYP4A mRNA levels observed after feeding of the fasted animals. Both 19- and 20-HETE formation were decreased in the 24- and 48-h groups (Table II). Feeding decreased arachidonic acid ω-hydroxylation 35% and (ω-1)-hydroxylation 35–50%. Epoxide activity was decreased 65% in the 24-h treatment group but was similar to control in the 48- and 72-h treatment groups.

CYP4A-immunoreactive protein levels were increased in the livers of animals fasted for 24–72 h (Fig. 5). The induction was maximal at 48 h and remained at this level following a 72-h fast. In these samples three immunoreactive protein bands were detected. The fastest migrating protein was tentatively identified as CYP4A1 based on the previous characterization of this antibody, and it was induced to a greater degree than the other two CYP4A isoforms (37). This is consistent with the greatest effect on CYP4A1 mRNA levels. Despite a dramatic decrease in CYP4A mRNA levels after feeding animals that had previously been fasted, the corresponding protein levels were still induced relative to control in these livers. The level of induction was less than that in the fasted livers and showed a gradual decrease as the fasting and feeding period increased in length. As in the fasted livers, the putative CYP4A1 protein was affected to the greatest degree.

**Time Course of CYP4A Suppression by Feeding**—The dramatic decrease in CYP4A mRNA levels after restoring access to food following a fasting period was surprising and suggested distinct regulation of the CYP4A mRNAs and proteins under these conditions. This was investigated further by following the time course of this suppression. Rats were fasted for 72 h after which they were allowed free access to food for a period of 24–96 h. Analysis of the CYP4A mRNA levels in the livers of these animals showed the expected modest increase in CYP4A mRNA levels after the fasting period and showed a rapid decline in CYP4A mRNA levels after reintroduction of food (Fig. 6). Within 24 h of feeding, CYP4A mRNA levels were maximally reduced to 20–35% of control values. After 72 h of feeding CYP4A mRNA levels showed signs of gradual recovery and were 45–55% of control 96 h after the end of the fasting period. In contrast, both lauric acid and arachidonic acid ω- and (ω-1)-hydroxylase activity returned to control values within 24 h of ending the fasting period (data not shown). CYP4A-immunoreactive protein levels remained at induced levels for the entire 96 h following the fast and showed gradual signs of returning toward control values (Fig. 7).

**Induction of AOX by Diabetes and Fasting**—To explore whether the inductive effects of diabetes and fasting were specific for the CYP4A genes, we also characterized the expression of AOX in these samples. Both diabetes and fasting induced hepatic AOX expression (Fig. 8). The inductive effect of diabetes on hepatic AOX expression was modest and was not completely reversed by insulin treatment. Following a 48-h fast...
hepatic AOX expression was induced severalfold. Similar to the effects on CYP4A expression, refeeding of fasting animals reduced AOX expression to levels that were below those observed in the control animals.

**Induction of CYP4A by Diabetes and Fasting Is Mediated through PPARα**—Mice lacking PPARα were used to characterize the role of this receptor in the induction of the CYP4A genes by diabetes and fasting. C57BL/6 control mice and PPARα+/− mice were made diabetic with streptozotocin. Induction of diabetes was evident in both the C57BL/6 and PPARα−/− mice from the blood glucose levels measured 10 days after streptozotocin treatment. Blood glucose levels were 94.8 ± 39.1 and 166 ± 28.7 mg/dl in the vehicle-treated PPARα+/− and C57BL/6 mice, respectively. In the streptozotocin-treated group, blood glucose levels increased to 374 ± 137 mg/dl in the PPARα−/− mice and to 477 ± 17.2 mg/dl in the C57BL/6 mice. Constitutive expression of the CYP4A genes was low in both the C57BL/6 and the PPARα−/− mice (Fig. 9). In the control mice, diabetes was associated with a dramatic increase in CYP4A mRNA levels, and this induction was completely absent in the PPARα−/− mice.

A 30-h fasting period resulted in a significant decrease in blood glucose and a 10% loss of body weight, both of which were completely reversed by feeding. Blood glucose levels dropped from 138 ± 15.6 mg/dl in fed C57BL/6 mice to 65.6 ± 4.9 mg/dl in fasted C57BL/6 mice. In the PPARα−/− mice, blood glucose levels decreased from 84.6 ± 27.7 mg/dl in the fed animals to 37.2 ± 4.2 mg/dl in the fasted animals. Fasting induced a dramatic increase in hepatic CYP4A mRNA levels in the C57BL/6 mice which was reversed after feeding these animals for 42 h following the fast (Fig. 10). Induction of PPARα-responsive genes by fasting was not restricted to CYP4A. Both the BIEN and thiolase genes were also induced by fasting in the livers of diabetic C57BL/6 mice, and this response was reversed after feeding these animals following the fast. In contrast, there was no induction of CYP4A4, BIEN, or thiolase in the livers of the PPARα−/− mice. Thus, *in vivo* induction of these genes by fasting strictly requires a functional PPARα.
increased activation of PPAR during both diabetes and starvation may be responsible for the triacylglycerol hydrolysis in the liver (38, 39). These increased from adipose tissue, inhibition of glucose uptake by muscle, and blood (38, 39). Likewise, the decreased blood glucose levels within the hepatocyte as well as the muscle, adipose tissue, and body formation, thereby increasing the levels of free fatty acids hydrolysis, fatty acid oxidation, gluconeogenesis, and ketone associated with diabetes cause an increase in triacylglycerol receptor, as both diabetes and starvation are associated with functional support for fatty acids as endogenous activators of this nuclear receptor. It has been widely accepted that fatty acids can transcriptionally activate PPAR in in vitro transactivation assays (19–21). Recently, it was demonstrated that fatty acids are indeed ligands for this receptor, providing strong evidence that they are endogenous PPAR activators (22, 23). The current study provides additional support for fatty acids as endogenous activators of this receptor, as both diabetes and starvation are associated with increased levels of free fatty acids. High blood glucose levels associated with diabetes cause an increase in triglyceride hydrolysis, fatty acid oxidation, gluconeogenesis, and ketone body formation, thereby increasing the levels of free fatty acids within the hepatocyte as well as the muscle, adipose tissue, and blood (38, 39). Likewise, the decreased blood glucose levels associated with fasting result in a mobilization of fatty acids from adipose tissue, inhibition of glucose uptake by muscle, and triglyceride hydrolysis in the liver (38, 39). These increased levels of free fatty acids in the circulation and in the liver during both diabetes and starvation may be responsible for the increased activation of PPARα and CYP4A induction observed in the present study. Induction of the CYP4A enzymes will lead to changes in fatty acid metabolism and therefore in the levels of putative PPAR activators, suggesting a regulatory mechanism for the transcription of genes encoding lipid-metabolizing enzymes and transport proteins.

Diabetes was induced by a single injection of streptozotocin, a methylglyoxal derivative of glucose that has high affinity for β cells of the islets of Langerhans and shows selective toxicity to these cells (40). In the livers of diabetic animals there was a profound induction of the CYP4A genes with a coordinate increase in CYP4A mRNA, protein, and activity. Maintenance of normal blood glucose levels by the administration of insulin was able to reverse completely these effects. The induction is consistent with previous studies demonstrating increases in CYP4A protein levels and nonspecific levels of CYP4A mRNA following streptozotocin treatment (25–29). By using gene-specific RNase protection assays developed in our laboratory, we have clearly shown that all of the heptically expressed CYP4A genes are induced in the diabetic rat liver, with quantitative differences between the individual genes. The potential significance of this induction during diabetes is evident from the increase in arachidonic acid metabolism. Although limited information is available regarding the biological activities of CYP4A arachidonic acid metabolites in the liver, it is likely that 20-HETE may be involved in the regulation of vascular tone and ion transport as described previously in the kidney and brain (2). Increased long chain fatty acid ω-hydroxylation is also significant since it results in the generation of long chain dicarboxylic acids that uncouple oxidative phosphorylation. Long chain dicarboxylic acids are obligatory substrates for peroxisomal β-oxidation, and their metabolism leads to the formation of hydrogen peroxide and the possibility of oxidative stress and cellular damage (41).

Fasting was associated with even greater increases in CYP4A mRNA levels than those observed in diabetic livers. The effects of fasting on CYP4A expression were highly dependent on the length of the fasting period. Interestingly, although a 48-h fast resulted in as great as a 17-fold increase in CYP4A mRNA levels, a more prolonged fast of 72 h had a minimal effect. The return of blood glucose levels to control values in the 72-h fasted rats reflects an adaptive response to a prolonged fast and is associated with increases in liver glycogen and decreases in free fatty acids relative to the levels found at early times after initiating a fast (38, 39). Thus, the levels of the putative fatty acid activators of PPARα would be lower after the prolonged fast and are consistent with only minimal CYP4A induction (and therefore PPARα activation) in the 72-h fasted animals. Increases in CYP4A protein levels and lauric acid hydroxylation in the fasted livers were consistent with the changes in mRNA levels, although changes in protein lagged behind those in RNA. There is no information available on the half-life of CYP4A mRNA or protein, but this suggests a shorter half-life for the mRNA species. Although the effects of diabetes on CYP4A expression could be completely reversed by insulin treatment, allowing fasting animals free access to food resulted in a complex response that was discordantly regulated at the protein and RNA level. Feeding animals after a fasting period produced a significant reduction in CYP4A mRNA levels, whereas lauric acid ω-hydroxylation and CYP4A protein levels remained slightly elevated or returned to control values. Thus, the CYP4A protein levels seem to be much more tightly
controlled than the corresponding mRNA levels. This is also evident from the large difference between the relative increases in CYP4A mRNA levels in both diabetic and fasting livers and the accompanying changes in CYP4A protein levels or activity. Although CYP4A mRNA levels increase as much as 17-fold in the induced state, protein levels and functional activity increased only 2–3-fold. This tight regulation is consistent with the important function of the CYP4A enzymes in the formation of several important biological mediators from arachidonic acid.

Diabetes also modestly induced the epoxidation of arachidonic acid that is catalyzed by CYP2E1 and CYP2C23 (36, 42). Regulation of the CYP genes by insulin-dependent diabetes is widely recognized (27, 43, 44). The effect of diabetes on CYP2E1 expression is well characterized and has been shown to be mediated by the increased ketone levels that accompany this disorder (45, 46). The mechanistic basis for CYP2E1 induction by diabetes is stabilization of the mRNA (47), which is distinct from its induction by a number of small chemicals such as ethanol and isoniazid by protein stabilization (48). Thus, the increase in arachidonic acid epoxigenation likely reflects an induction of CYP2E1. Since CYP2E1 and CYP2C23 have also been implicated in the (ω-1)-hydroxylation of fatty acids, the increased formation of both 11-hydroxylauric acid and 19-HETE in diabetic rat liver microsomes may reflect an induction of not only the CYP4A proteins but also CYP2E1.

Fasting has also been associated with the induction of several other CYPs. The immunoreactive protein levels of CYP2E1, CYP3A2, CYP2B1, and CYP2A1 were increased in the livers of rats following a 48-h fast (30). In the case of CYP2E1, the increases in protein levels were accompanied by increases in activity and mRNA levels. Following a 72-h fast CYP2E1 mRNA levels increased 8–9-fold, whereas protein levels and β-nitrophenol hydroxylase activity increased 4–5-fold (49). Since arachidonic acid epoxigenase activity is catalyzed at least in part by CYP2E1, it seemed likely that this activity would be increased in the fasted livers in the present study; however, this was not apparent. This may reflect a minimal induction of CYP2E1 under the experimental conditions used in this study or alternatively may indicate that CYP2E1 plays only a minor role in the epoxidation of arachidonic acid.

Both diabetes and fasting coordinate induced all three of the hepatically expressed rat CYP4A genes. In both cases CYP4A1 showed the largest increase in mRNA levels that partially reflects its low constitutive expression. Coordinate induction of the hepatic CYP4A genes has also been demonstrated for the chemical inducers clofibrate, dehydroepiandrosterone, and diethylhexylphthalate (11, 29, 50, 51). Clofibrate showed preferential induction of CYP4A1, inducing CYP4A1 mRNA levels 39-fold in male rat liver microsomes and CYP4A2 and CYP4A3 levels only 8–10-fold (50). Collectively, these data argue for a common mechanism of inducible regulation of the CYP4A genes. It is still not clear whether coordinate regulation also exists for the renal CYP4A genes. Treatment with clofibrate induced the hepatic CYP4A genes in a coordinate fashion, whereas only CYP4A1 and CYP4A3 were significantly induced in the kidney (29). In contrast, cyclosporin A induced only the CYP4A2 isomorph in rat renal microsomes (52). There is limited evidence that CYP4A2 protein levels are increased in the kidneys of diabetic and fasted rats (53, 54). In light of the importance of the CYP4A-mediated eicosanoids in the regulation of renal function and blood pressure, the effects of diabetes and fasting on the expression of the renal CYP4A genes is a compelling question. It will also be important to determine whether PPARα is necessary for the induction of the renal CYP4A genes by these pathophysiological conditions.

The CYP4A enzymes are regarded as fatty acid and prostaglandin ω-hydroxylases, although the substrate specificity and kinetic constants for the individual rat isoforms have not been clearly defined. The ω-hydroxylation of lauric acid and arachidonic acid was similarly induced in the diabetic rat livers and showed changes consistent with the increase in CYP4A mRNA and protein. In the fasted livers, lauric acid more accurately reflected the changes in CYP4A mRNA and protein levels than did arachidonic acid. We have previously shown in the rat kidney that lauric acid ω-hydroxylation is more tightly linked to CYP4A mRNA and protein levels than is arachidonic acid ω-hydroxylation (32). The difference in activity with these two fatty acids may reflect the difference in abundance of the CYP4A isoforms in the rat liver and their relative importance in the metabolism of arachidonic versus lauric acid. This is supported by functional data on the rabbit CYP4A enzymes. Rabbit CYP4A7 showed the highest activity for both arachidonate (ω-6)-hydroxylation and lauric acid ω-hydroxylation. However, significant laurate ω-hydroxylation was also observed with CYP4A5 which metabolized arachidonic acid at negligible rates (4). The determination of whether a similar situation exists for the rat CYP4A enzymes requires a more complete characterization of these proteins.

The requirement for a functional PPARα to transactivate CYP4A genes in response to diabetes or starvation is highly evocative of a biological activation of this receptor by physiological activators that remain to be fully characterized. Induction of the CYP4A enzymes, major fatty acid and prostaglandin ω-hydroxylases, may represent a regulatory mechanism for control of the hepatic concentrations of putative endogenous fatty acid activators of PPARα, thereby controlling the transcription of a number of genes involved in lipid metabolism and transport. The activation of PPARα during fasting and diabetes induces not only the CYP4A genes but also other PPARα-responsive genes, including acyl-CoA oxidase, enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase which encode enzymes involved in fatty acid β-oxidation. It is likely that additional PPARα-responsive genes such as acyl-CoA synthase that converts fatty acids into acyl-CoA derivatives, liver, and adipocyte fatty acid-binding proteins important for intracellular transport of long chain fatty acids, phosphoenolpyruvate carboxykinase which is necessary for gluconeogenesis, and HMG-CoA synthase involved in liver ketogenesis are also induced during these pathophysiological conditions (14). Thus we can speculate that activation of PPARα during diabetes and starvation is an important mediator of the changes in fatty acid metabolism and transport and intracellular energy balance during these clinical conditions. Alterations in lipid transport and storage may also be mediated through PPARα since the apolipoprotein A-I and A-II genes are also induced by peroxisome proliferators and are likely to be activated during diabetes and starvation. Further studies into the metabolic consequences of PPARα activation during these clinical conditions is clearly warranted.

In summary, we have shown that insulin-dependent diabetes and starvation result in induction of the hepatic CYP4A genes and other lipid-metabolizing enzymes through activation of the PPARα. This constitutes the first demonstration that the requisite cellular changes necessary for signaling of a member of the nuclear receptor family can be produced in a pathophysiological state. Based on the recent data that identified long chain fatty acids as ligands of the PPARα, it seems plausible that the increased fatty acid levels associated with diabetes and starvation are involved in the activation of this receptor. Future investigations to identify the individual fatty acids involved in this activation will further bridge the gap between
our mechanistic understanding of PPARα activation and its functional significance in vivo. Increased arachidonic acid ω-hydroxylation resulting from induction of the hepatic CYP4A genes may have profound effects on the physiological role of this metabolite in the regulation of vascular tone and ion transport and illustrates the potential importance of PPARα activation in vivo. The physiological significance of CYP4A induction, especially during chronic uncontrolled diabetes mellitus, warrants further investigation.

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