In vivo involvement of cytochrome P450 4A family in the oxidative metabolism of the lipid peroxidation product *trans*-4-hydroxy-2-nonenal, using PPARα-deficient mice

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Abstract *Trans*-4-hydroxy-2-nonenal (HNE) is a potent cytotoxic and genotoxic compound originating from the peroxidation of n–6 polyunsaturated fatty acids. Its metabolism has been previously studied in the rat (Alary et al. 1995. *Chem. Res. Toxicol.*, 8: 35–39). In addition to major urinary mercapturic derivatives, some polar urinary metabolites were isolated and could correspond to hydroxylated compounds. 4-Hydroxynonenoic acid (HNA), resulting from the oxidation of the HNE carbonyl group, is a medium chain fatty acid and its ω-hydroxylation might be hypothesized. Therefore, the involvement of the CYP 4A family isoenzymes in the metabolism of [³H]HNE has been investigated in vivo using inducer treatments (fibrates) in wild-type or in peroxisome proliferator-activated receptor α (PPARα)-deficient mice. In wild-type mice, but not in PPARα (−/−) mice, fibrate treatments resulted in an increase of two urinary metabolites characterized, after HPLC purifications and mass spectrometry analyses, as the ω-hydroxylated metabolite of HNA, i.e., 4,9-dihydroxy-2-nonenoic acid, and its oxidized form, 4-hydroxy-2-nonene-1,9-dicarboxylic acid. The formation of the latter is correlated accurately to laurate hydroxylase activity studied concurrently in microsomes prepared from the liver of these animals. Basal levels of these two metabolites were measured in urine of normal and PPARα-deficient mice.—These results are in accord with an implication of the P450 4A family in the extended oxidative metabolism of 4-HNE.—Guéraud, F., J. Alary, P. Costet, L. Debrauwer, L. Dolo, T. Pineau, and A. Paris. **In vivo involvement of cytochrome P450 4A family in the oxidative metabolism of the lipid peroxidation product *trans*-4-hydroxy-2-nonenal, using PPARα-deficient mice.** *J. Lipid Res.* 1999. 40: 152–159.

Supplementary key words   4-HNE • PPARα • knockout mice • CYP 4A • ω-hydroxylation

Trans-4-hydroxy-2-nonenal (HNE), an endogenous α,β-unsaturated aldehydic product resulting from lipid peroxidation (1) is considered to be a potent cytotoxic and genotoxic compound as evidenced by in vitro studies (2, 3). Its important electrophilic nature explains the chemical reactivity towards thiols (4, 5), amino groups of amino acids and proteins (lysine, histidine) (6–8), or DNA bases (deoxyguanosine) (9, 10). In vivo, a substantial level of oxidoreductive metabolism associated with glutathione (GSH) conjugation has been evidenced in the rat (11). A rapid decrease in the HNE pool was observed, resulting in urinary alcohol, acid, and lactone mercapturic derivatives (11). As previously mentioned by Mitchell and Petersen (12) and Esterbauer, Zollner, and Scholz (13), cytosolic aldehyde and alcohol dehydrogenases seem to be involved in the oxidation and reduction of HNE, respectively, the resulting product being likely processed by a subsequent conjugation to GSH (11). Yet, in this previous in vivo metabolic study of [4-³H]HNE in the rat, almost 40% of the urinary radioactivity was shown to belong to high polarity metabolite classes and remained unidentified to date.

4-Hydroxynonenoic acid (HNA) resulting from the oxidation of HNE belongs to the medium chain fatty acid class. A further oxidative metabolism of HNA mediated by cytochromes P450 4A gene family could be then hypothesized. Noticeably, among other mammalian cytochrome P450s, these isozymes display the specific ability to sustain the ω- and (ω–1)-hydroxylation of medium and long chain fatty acids as well as prostaglandins and leukotrienes (14). Hepatic levels of cytochrome P450 4A isoenzymes are increased after induction by peroxisome proliferators (15–17). Such an induction by fibrates is mediated by the peroxisome proliferator-activated receptor α (PPARα), a member of the nuclear receptor family of ligand-activated transcription factors (18–20). In addition, the targeted

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disruption of the mouse PPARα gene prevents the proliferation of peroxisomes and the subsequent induction of cytochrome P450 4A isoenzymes after exposure of genetically engineered animals to peroxisome proliferators (21).

In the present study, biochemical and analytical evidences that ω-hydroxylation of HNA is mediated in vivo by cytochrome P450 4A isoenzymes are given using fibrate induction treatments in wild-type as well as in genetically PPARα-deficient mice.

MATERIALS AND METHODS

Chemicals

HNE diethyl acetal was synthesized according to Esterbauer and Weger (22). [4-^{3}H]HNE diethyl acetal was synthesized at CEA (Service des Molécules Marquées, CEN, Saclay, France), according to the method developed for the deuterated compound (23). Its radiochemical purity, determined by HPLC, was 95%, and its specific activity was 222 GBq/mmol. HNE was prepared from its ethyl acetal derivative (stored in chloroform) just prior to use by 1 mm HCl hydrolysis for 1 h at room temperature. The hydrolysis was followed by extraction of HNE with CH_2Cl_2 and evaporation of the solvent. HNE concentration in water was measured spectrophotometrically at 223 nm. HNA was synthesized from HNE as described before (11). [4-^{3}H]HNA was synthesized from [4-^{3}H]HNE in the same way. [1-^{14}C]lauric acid (2.15 GBq/mmol) was purchased from Amersham (Les Ulis, France).

Clofibrate was purchased from Sigma (St-Quentin Fallavier, France). Fenofibrate was a generous gift from Fournier (Dijon, France). All solvents and reagents for the preparation of buffers and HPLC eluents were the highest grade commercially available from Sigma, Prolabo (Fontenay-sous-Bois, France), Scharlau (Ferosa, Barcelona, Spain), and Merck (Nogent-sur-Marne, France). Ultrapure water from Milli-Q system (Millipore, St-Quentin-en-Yvelines, France) was used for HPLC eluent preparation.

Animal treatment

Care of mice was according to institutional guidelines. PPARα (−/−) mice originated from homologous recombinant 129Sv-derived cells as described before (21). Chimeric males were initially backcrossed to C57BL/6 females. Several additional rounds of backcrossing were performed to increase the C57BL/6 genetic background and to generate the animals used in this study.

Conventional age-matched C57BL/6 female mice were obtained from Iffa-Credo (L'arbresle, France). During fibrate treatment, mice were fed ad libitum on a standard laboratory diet (UAR, AO3, Epinay-sur-Orge, France) and housed in groups of three in plastic cages at 25°C with a 14-h light/10-h dark cycle. Mice received clofibrate (400 mg/kg body wt per day in groundnut oil) and fenofibrate (100 mg/kg body wt per day suspended in a 3% aqueous solution of arabic gum) by gavage daily for 3 days. Respective control animals received the appropriate vehicle. Twenty-four hours after the end of this fibrate treatment, animals were individually housed in metabolic cages with fritted glass ground allowing urine/feces separation and injected intraperitoneally with radiolabeled 4-HNE (10 mg/kg body wt in saline; specific activity: 455 MBq/mmol). Animals were killed by cervical dislocation 24 h after 4-HNE injection. The liver was rapidly removed, weighed, and snap frozen in liquid nitrogen before storage at −80°C.

A microsomal fraction was prepared for each animal as previously described (24).The amount of microsomal protein was determined by the method of Lowry et al. (25) using BSA as a standard. Urine was eluted under vacuum from a fritted glass ground cage with 100 ml distilled water and stored at −20°C. Twenty ml of this eluate was used for subsequent urinary 4-HNE metabolite analysis. Preliminary experiments have shown that most of the intraperitoneally administered radioactivity is excreted in urine (40–60%), less than 2% being excreted in feces.

HPLC analyses of HNE metabolites

Before HPLC analysis, diluted urine was filtered under vacuum through a 0.45-μm HA Millipore membrane and then concentrated using a Supelclean LC18 SPE 1 g cartridge (Supelco inc., Bellefonte, PA) after acidification to pH 2–3 with 1 m phosphoric acid. The cartridge was washed with 10 mL 0.01 m phosphoric acid solution at pH 2–3, dried under a nitrogen stream, and HNE metabolites were eluted with 5 mL methanol. Part (7–16%) of the urinary radioactivity was unretained on the cartridge. A 0.5-mL aliquot of the methanolic elution was evaporated under nitrogen and analyzed by HPLC for metabolite separation on a Supelco LC18 column (25 × 4.6 mm i.d., 5 μm particle size) (Supelco Inc.) with a Philips model PU4100 apparatus (Argenteuil, France) equipped with a Rheodyne Model 7125 injector (Rheodyne, Cotati, CA), a 500 μL loop and connected to an on-line radioisotope detector Radiomatic Flo-One/beta model A515 (Packard, Meriden, CT). The HPLC system 1 was used with solvents A [ammonium acetate (20 mm, pH 4.5)–acetonitrile 97.5:2.5 (v/v)], B [ammonium acetate (20 mm, pH 4.5)–acetonitrile 80:20 (v/v)], and C [ammonium acetate (20 mm, pH 4.5)–acetonitrile 30:70 (v/v)] as follows: from 0 to 4 min, 100% A; from 4 to 5 min, linear gradient from 100% A to 85.7% A/14.3% B; from 5 to 25 min, linear gradient from 85.7% A/14.3% B to 80% A/20% B; from 25 to 35 min, 80% A/20% B; from 35 to 40 min, linear gradient from 80% A/20% B to 100% B, which is maintained from 40 to 45 min; from 45 to 50 min, linear gradient from 100% B to 100% C, then maintained from 50 to 60 min, the whole run being achieved at a flow rate of 1 mL/min at 35°C. The HPLC system 2 used the same elution gradient as the HPLC system 1 on a semipreparative column, Shandon Ultrabase C18 (250 × 7.5 mm i.d., 5 μm particle size) (SFCC, Eragny, France), with a 2 mL loop, at a flow rate of 2 mL/min at 35°C. The HPLC system 3 was performed on the same analytical column as for the HPLC system 1 at a flow rate of 1 mL/min at 35°C and lasted 60 min. The elution gradient was used as follows: from 0 to 5 min, 100% A [water–acetonitrile–acetic acid 97.5:2.5/1 (v/v/v), from 5 to 30 min, linear gradient from 100% A to 85.7% A/14.3% B [water–acetonitrile–acetic acid 80:20:1 (v/v/v)], from 30 to 40 min, linear gradient from 85.7% A/14.3% B to 80% A/20% B, which is then maintained for 5 min; from 45 to 46 min, linear gradient from 80% A/20% B to 50% A/50% B, which is finally maintained for 14 min.

Mass spectrometry analyses

The two peaks displaying a quantitative increase related to fibrate treatments (peaks A and B in HPLC system 1, eluted at 13.5 and 15.0 min, respectively) were quantitatively purified from the urine of one clofibrate-treated mouse. It was done by using the preparative HPLC system 2 and a Gilson model 202 fraction collector (Gilson Medical Electronics, Viliers-le-Bel, France). Part (5–10%) of the eluted radioactivity was submitted to liquid scintillation counting in a 2200CA Packard spectrophotometer (Packard, Downers Grove, IL) using Ultimagold™ (Packard) as scintillation cocktail. The collected fractions were further purified using HPLC system 3 before being submitted to mass spectrometry analyses. The dry compounds were methylated for 30 min at room temperature by addition of 50 μL etheral diazomethane. Then, solvent was removed under a nitrogen stream

and the dry residue was subsequently dissolved in a mixture of N,O-bis(TMS)-trifluoroacetamide–trimethylchlorosilane (Pierce Chemical, Rockford, IL) in a 99:1 ratio and heated at 60°C for 1 h. The solvent was evaporated at 30°C under a nitrogen stream and hexane (20 μL) was added to the dry residue. For GC–MS analyses a Nermag R-10-10-T single quadrupole instrument was coupled to a Delsi DI 200 (Delsi Nermag Instruments) gas chromatograph with a BPX5 (25 m × 0.22 mm × 0.25 μm) capillary column (SGE, Villeneuve-St-Georges, France). The samples (1 μL) were injected in the splitless mode. Helium was used as the carrier gas at a flow rate of 1 mL/min with a back pressure of 0.8 bar. The oven temperature was set as follows: 50°C for 50″s, then from 50°C to 230°C at 25°C/min, and finally from 230 to 270°C at 5°C/min. The injector temperature was 270°C and the interface temperature was 270°C. Electron impact (EI) mass spectra were generated at 70 eV with an emission current of 200 μA at a source temperature of 220°C.

Assay of P450 4A mRNAs

Total RNA was isolated from each liver by acid-phenol extraction. Construction of a specific probe for rat P450 4A3 was done as described previously (16, 26). Northern blot analysis of wild-type (+/+) and PPARα homozygous mutant (−/−) mice treated with either clofibrate or fenofibrate were done as described elsewhere (21). The results indicated that PPARα-deficient mice displayed no induction of P450 4A mRNA expression with any of the fibrate treatments used, while wild-type mice presented a great increase in this expression with both treatments (results not shown) as it was pointed out in a previous study (21).

Lauric acid ω- and (ω-1)-hydroxylation activities

Lauric acid ω- and (ω−1)-hydroxylation activities were measured with [1-14C]lauric acid (sp act: 24 MBq/mmol). Reactions mixtures were prepared as described by Orton and Parker (27). Incubations lasted 30 min at 37°C with 2 mg microsomal protein. The hydroxylauric acids and unreacted substrate were extracted into diethylether and evaporated to dryness. The formation of 11- and 12-hydroxylauric acids was assayed by HPLC on a Spherisorb C18 column (25 × 4.6 mm i.d., 5 μm particle size) maintained at 35°C with a Philips model PU 4100 apparatus equipped with a 500 μL loop and an on-line radioisotope detector Radiomatic Flo-One/beta model A515. The mobile phase was a mixture of acetonitrile and 0.1 m ammonium acetate buffer (pH 4.6) (35:65, v/v) for 15 min, followed by 100% acetonitrile for another 15 min (28). The flow rate was 1 mL/min.

Immunoinhibition

Anti-rat P450 4A1 serum was manufactured by Daiichi Pure Chemicals Co., Ltd (Tokyo, Japan).This serum contains a polyclonal antibody made for studying the inhibition of P450 4A1 catalyzed enzyme activity. Microsome incubations were done according to the supplier's recommendations with 25 μm radiolabeled [4-3H]HNA as substrate (sp act: 680 MBq/mmol). Microsomal proteins (200 μg) were used for preliminary clofibrate-treated rat studies. These studies were achieved to verify the antibody effect with HNA as substrate. Microsomal proteins (500 μg) were used for clofibrate-treated mouse studies. Incubations were stopped by 4 vol methanol. After centrifugation, the liquid phase was submitted to HPLC using the same equipment as for lauric acid hydroxylation studies. The mobile phase was a mixture of acetonitrile and 20 mm ammonium acetate buffer (pH 4.6) (15:85, v/v) for 15 min, then (30:70, v/v) for another 15 min. The flow rate was 1 mL/min.

Statistical analyses

Treatment effects were submitted to variance analyses and comparisons of means were done with the Student-Newman-Keuls procedure. Regression analyses were made using the classical linear model. All statistical analyses were done using SAS software (29).

RESULTS

Effect of fibrate induction on the excretion of polar metabolites of [³H]HNE in urine

Urinary metabolites of [4-3H]HNE were quantified in mice by radio-HPLC using the HPLC system 1. Two main metabolites were separated and identified as mercapturic conjugates of 4-hydroxy-2-nonenoic (HNA-MA) and 1,4-dihydroxy-2-nonene (DHN-MA) eluted respectively at 28.7 and 32.4 min (**Fig. 1**) by comparison with rat urinary metabolites as recently published (11). In control animals, the proportion of polar metabolites eluted between 5.0 and 18.0 min was about 18.0% of the total radioactivity administered. Independent of the peroxisome proliferator administered, the effect of induction was very significant ($P < 0.01$) on the excretion of polar metabolites of [³H]HNE in urine of treated wild-type mice (**Fig. 2A**) as compared with respective control animals. We observed 28.8 and 35.9% increases in polar metabolites excretion with clofibrate and fenofibrate treatments, respectively. Noticeable modifications in the ratio of less polar metabolites were not observed at the same time. Polar metabolites were separated into different peaks that contained unresolved complex mixtures of compounds. Two peaks (A: Rt = 13.3 min and B: Rt = 15.0 min, Fig. 1) were specifically increased after clofibrate or fenofibrate treatments (Figs. 2B and 2C). When compared to controls, peak A was increased by 80.3% and 94.7% ($P < 0.001$) by clofibrate and fenofibrate treatments, respectively. The increase in urinary excretion of metabolites eluted under peak B was also significant: 22.8% ($P < 0.02$) and 78.8% ($P < 0.001$) for clofibrate and fenofibrate treatments, respectively. Furthermore,
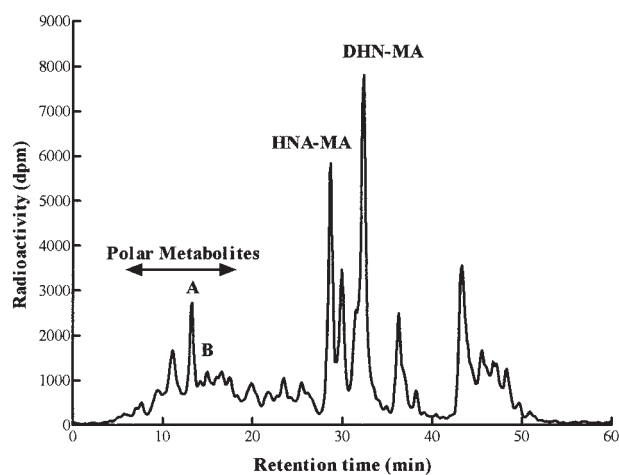
Fig. 1. Separation on the HPLC system 1 of the urinary metabolites of [4-3H]HNE collected for 24 h and purified from one clofibrate-induced mouse. HNA-MA and DHN-MA are, respectively, the mercapturic derivatives of 4-hydroxy-2-nonenoic acid and 1,4-dihydroxy-2-nonene.
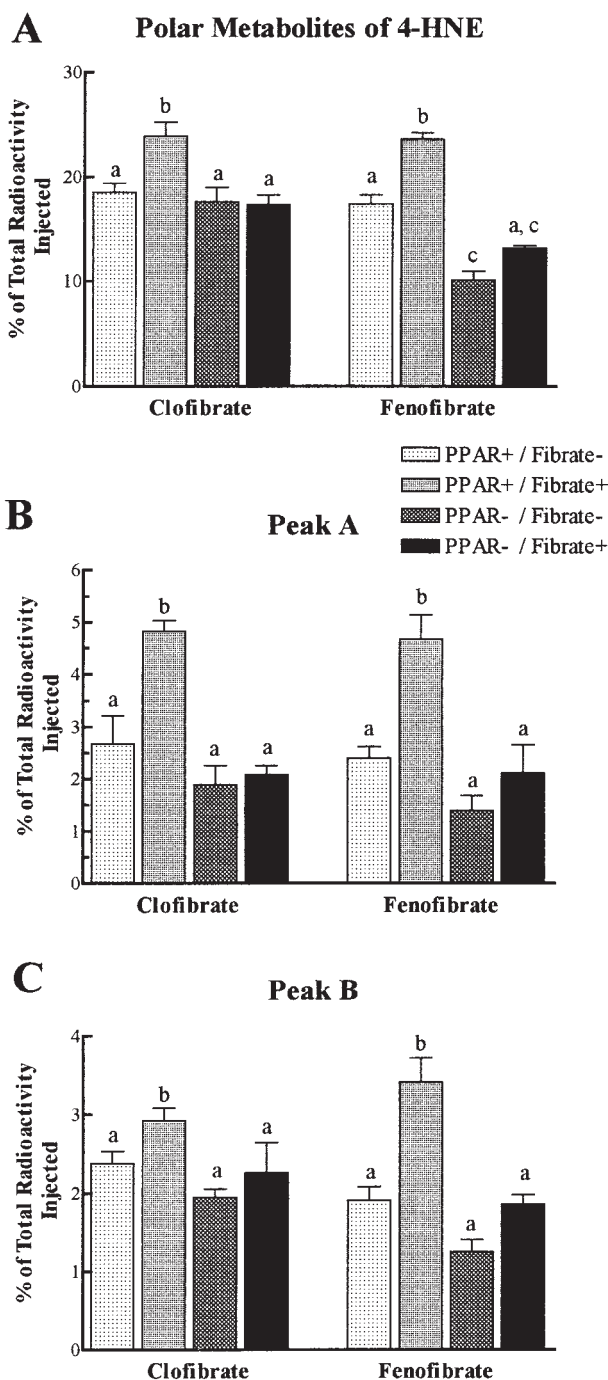
A
Polar Metabolites of 4-HNE

Legend:
- ▢ PPAR+ / Fibrate−
- ▨ PPAR+ / Fibrate+
- ▨ PPAR− / Fibrate−
- ■ PPAR− / Fibrate+

B
Peak A

C
Peak B

Fig. 2. Quantification of urinary polar metabolites of HNE (A), peak A (B), and peak B (C) measured by radio-HPLC using the system 1 as a function of fibrate treatment and animal model.

neither peaks A and B nor the total proportion of polar metabolites displayed any increase with fibrate-treatments in control or fibrate treated PPARα-deficient mice (Figs. 2A, 2B, and 2C). Altogether, these results indicate that the relative increase in the amounts of urinary metabolites eluted under peaks A and B after treatment with clofibrate or fenofibrate is linked to a metabolic process related to an enzymatic induction mediated by PPARα.

Isolation and identification of 4-hydroxy-2-nonene-1,9-dicarboxylic acid and 4,9-dihydroxy-2-nonenoic acid

The group of polar metabolites eluting at 13.3 min (peak A) in the analytical HPLC system 1 was purified in a larger amount by a semi-preparative method (system 2). These metabolites were further purified on an analytical column using HPLC system 3. A major metabolite (Met-A3), which represents about 36% of the radioactivity belonging to peak A, was separated from numerous minor metabolites (**Fig. 3**). After methylation and silylation, the metabolite was analyzed by GC-MS using electron impact ionization. A peak was detected at 9.3 min. It shares a fragment ion at m/z 187 with HNA when analyzed in the same conditions. This fragment could correspond to the following ion: $[(CH_3)_3Si-O = CH-CH = CH-COOCH_3]^+$ (**Fig. 4A**) and could be interpreted as the result from the α-cleavage of the O-TMS group in position 4. Other relevant fragment ions can be explained as follows: m/z 287 [M–$CH_3]^+$, m/z 270 [M–$CH_3OH]^+$, m/z 239 [M–CH_3OH–$CH_3O]^+$, m/z 229 [M–$CH_2COOCH_3]^+$. The absence of any ion at m/z 147 $[(CH_3)_3Si=O-Si(CH_3)_3]^+$ rules out the presence of two silylated hydroxy groups for this metabolite, but fragment ions at m/z 75 $[(CH_3)_2Si-OH]^+$ and m/z 73 $[(CH_3)_3Si]^+$ correspond to at least one hydroxy group. Besides, the presence of fragment ions at m/z 239 and m/z 229 is indicative of two acidic functions. On the basis of these data, this metabolite may be identified as 4-hydroxy-1,9-dicarboxy-2-nonene.

As described above, metabolites belonging to peak B were quantitatively purified by semipreparative HPLC. A metabolite eluting at 28.5 min in HPLC system 3 (not shown) was subsequently methylated and silylated before mass spectrometry analysis. When submitted to GC-MS analysis, a metabolite was detected at 9.4 min. As for the methylated and silylated derivative of 4-hydroxy-1,9,dicarboxy-2-nonene, a fragment ion at m/z 187 was detected and could correspond to the same structure: $[(CH_3)_3Si-O =CH-CH=CH-COOCH_3]^+$. This ion clearly represents
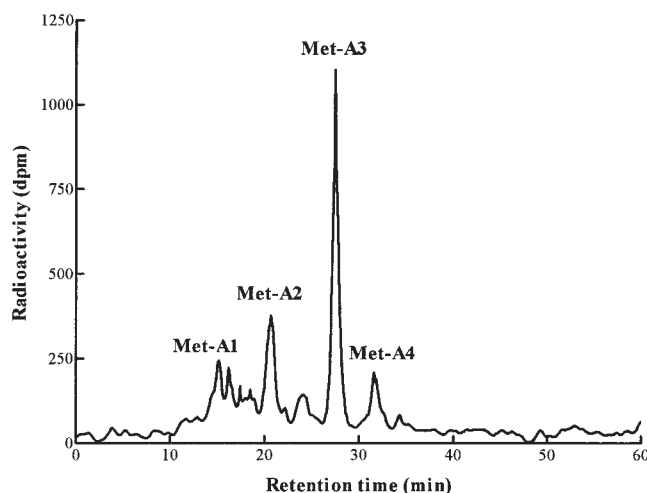
Fig. 3. Repurification on the HPLC system 3 of the metabolites belonging to the peak A purified on the HPLC system 1. Met-A3 is specifically increased by fibrate induction of wild-type mice.
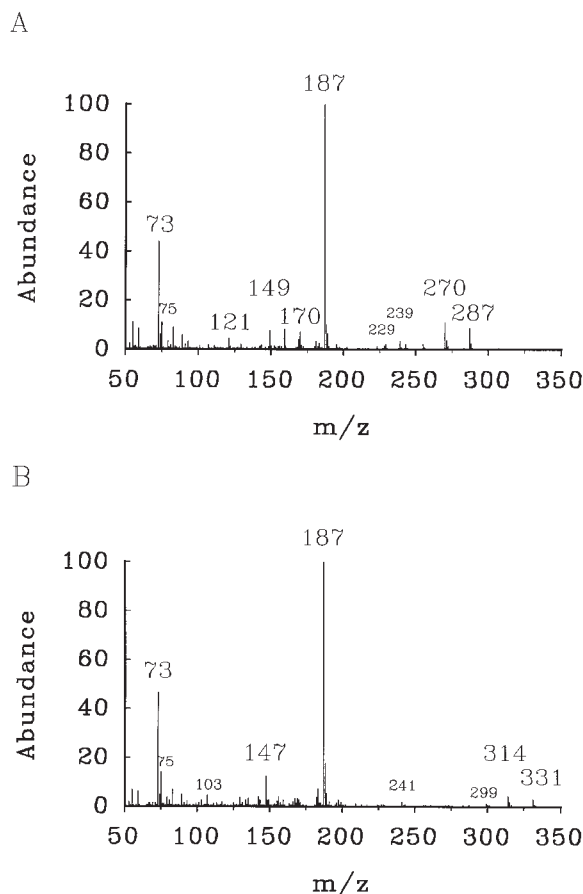
A

B

Fig. 4. Mass spectra obtained by GC/MS of 4-hydroxy-2-nonene-1,9-dicarboxylic acid (A) and 4,9-dihydroxy-2-nonenoic acid (B).

the α-cleavage of the 4-O-TMS group (Fig. 4B). The fragment ions at m/z 75 $[(CH_3)_2Si–OH]^+$ and m/z 73 $[(CH_3)_3Si]^+$ may correspond to at least one hydroxy group. Moreover, a fragment ion at m/z 147 $[(CH_3)_3Si=O–Si(CH_3)_3]^+$ is characteristic of the presence of two silylated hydroxy groups corresponding to two alcohol functions. However, the bis-TMS methylester of an hypothesized dihydroxy-2-nonenoic acid (m/z 346) was not detected. Several fragment ions can bring further evidence for such a structure: m/z 331 $[M–CH_3]^+$, m/z 314 $[M–CH_3OH]^+$, m/z 299 $[M–CH_3–CH_3OH]^+$, m/z 241 $[M–CH_3–(CH_3)_3Si–OH]^+$. Last, a minor but significant fragment at m/z 103 $[(CH_3)_3Si–O=CH_2]^+$ would constitute another element in favor of hydroxylation on C-9. These data demonstrate the presence of an ω-hydroxylated derivative of HNA that is in complete agreement with the fragmentation pattern observed for ω-hydroxy TMS ethers of fatty acid methyl esters (30).

Correlations with laurate hydroxylase activities

For each conventional or genetically deficient animal, the hepatic fatty acid ω-hydroxylation activity was measured in vitro on liver microsomes with lauric acid as substrate. Concurrently, the urinary radioactivity was analyzed in order to quantify the different metabolites of [4-³H]HNE, in respect to the fibrate-induction treatments.
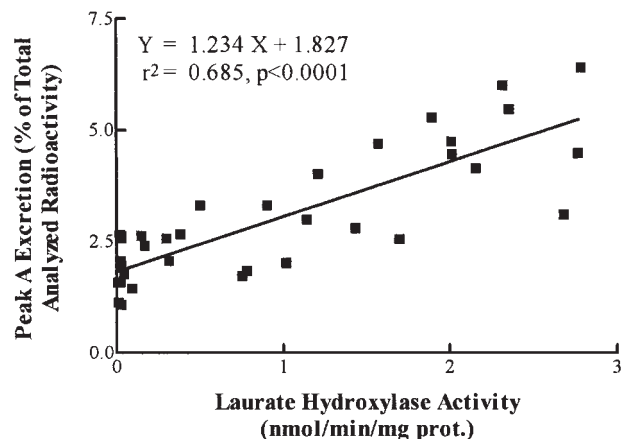

Fig. 5. Variation of the intensity of peak A excretion (in % of the total 24-h excreted urinary radioactivity analyzed by radio-HPLC on the system 1) as a function of the laurate hydroxylase activity measured on hepatic microsomes prepared after killing the same animals.

Due to a sufficient number of individuals (n = 34) and to a well-distributed set of values, it was possible to calculate a linear regression between the relative amount of radioactivity excreted under peak A (Y) and the laurate hydroxylase activity (X) without using any weighing in the regression calculation process (**Fig. 5**). The relationship between Y and X is highly significant: the slope value is 1.234 ± 0.148 ($F_{1,32=}$ 69.64, $P < 0.0001$, $r^2 = 0.685$). The Y-intercept is significantly different from zero (95% interval confidence comprised between 1.42 and 2.24% of the total radioactivity analyzed by HPLC) and could be explained by the contribution of numerous minor metabolites having the same retention time as 4-hydroxy-2-nonene-1,9-dicarboxylic acid in the HPLC system 1. In fact, further analyses in the HPLC system 3 of peak A purified from urine of control or PPARα-deficient animals, for which ω-hydroxylation activities are supposed to be reduced to basal levels, revealed that 4-hydroxy-2-nonene-1,9-dicarboxylic acid can account for only 0.55 and 0.08% of total urinary metabolites of [4-³H]HNE, respectively.

Immunoinhibition

Preliminary incubations at low substrate concentrations (25 μm) with rat microsomes and anti-rat P450 4A1 antibody gave a 40% HNA ω-hydroxylation residual activity with 5 μl of serum and an almost complete inhibition with 8 μl. Incubation conducted in the same way with mouse microsomes and the same antibody gave a 40% HNA ω-hydroxylation residual activity with 25 μl of serum.

DISCUSSION

Gene invalidation by homologous recombination provide elegant in vivo tools of investigation for the assessment of the consequences of substracting specific genes. Applied to metabolic studies, these techniques may point out how specific genes are involved in the biotransformation of endogenous as well as xenobiotic compounds. De-

spite the difficulty in obtaining viable, healthy, and fertile mouse lines when applying knockout techniques (31), PPARα-deficient mice belong to those viable and fertile mouse lines (21). PPARα mediates the inductive effect of foreign chemical peroxisome proliferators on the expression of peroxisomal, mitochondrial, and microsomal enzymes involved in β-oxidation of fatty acids (18, 32). Particularly, PPARα, as a nuclear *trans*-activator, is involved in the hepatic induction of numerous genes, among which are cytochromes P450 4A, by peroxisome proliferators such as fibrates (19, 20, 33). This isoenzyme family is known to catalyse ω- and (ω–1)-hydroxylation of lauric acid, an exogenous medium chain fatty acid that represents the model substrate, but also of endogenous long chain fatty acids such as palmitic or arachidonic acid (34). HNA, resulting from the oxidation of the HNE aldehyde group, can be considered as an endogenous medium chain fatty acid. In a previous study in the rat, a group of polar metabolites of [^{3}H]HNE was shown to be present in a large amount in urine, about 40% of the analyzed radioactivity (11). Preliminary mass spectrometry analyses revealed an *m/z* increase of 16 as compared to corresponding less polar major metabolites (35). Consequently, some of these highly polar compounds could correspond to hydroxylated metabolites. This increase could be hypothetically explained by an ω-hydroxylation of the less polar metabolites of HNE such as HNA. As knockout models are not available in the rat, HNE metabolism was reassessed in the mouse with a specific focus on the variation in the relative amount of polar metabolites. In a preliminary study, it was shown that similar groups of metabolites, with the same major metabolites, i.e., HNA-MA and DHN-MA, could be found in an almost similar proportion as those described previously in rats (11). Allowing the use of PPARα-knockout and wild-type mice, animals were subjected to two different fibrate treatments in order to study, in vivo, the requirement of the receptor to relay the influence of fibrates on the HNE metabolism. As the two peaks A and B belonging to the highly polar metabolites were substantially increased with both fibrate treatments in wild-type mice but not in PPARα-deficient ones (Figs. 2B and 2C), it can be concluded that the nuclear receptor PPARα is necessary for the fibrate-dependent induction of some enzymes involved in the metabolism of HNE. Moreover, the peak A metabolite increase with fibrates is highly correlated with lauric acid hydroxylase activity (Fig. 5) and could correspond to an ω-hydroxylation product of HNE or one of its metabolites. When further quantitative purifications of peaks A and B were achieved, mass spectrometry analysis was made possible. Both major metabolites were unambiguously identified as 4-hydroxy-2-nonene-1,9-dicarboxylic acid and 4,9-dihydroxy-2-nonenoic acid (9-OH-HNA), respectively (Fig. 4). The diacid derivative resulted obviously from a further oxidation of 9-OH-HNA (**Fig. 6**). Immunoinhibition studies with microsomes showed that anti-rat P450 4A1 antibodies could prevent the HNA ω-hydroxylation in rat as well as in mouse. This body of proof confirms the fact that the P450 4A monooxygenase family, presumably a mouse isozyme that is orthol-
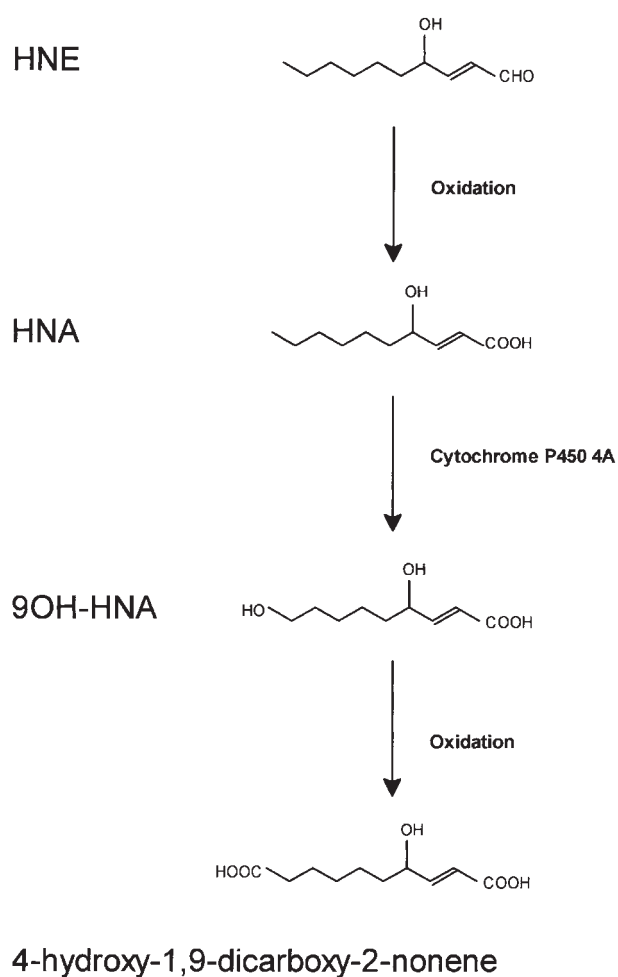

4-hydroxy-1,9-dicarboxy-2-nonene

Fig. 6. Oxidative metabolism of HNE implicating cytochrome P450 4A family.

ogous to rat P450 4A1 isozyme, is involved in the oxidative metabolism of lipid peroxidation product HNE in the mouse. These observations establish the first in vivo evidence that HNA, a medium chain fatty acid deriving from HNE by carbonyl oxidation, constitutes an endogenous substrate for the cytochrome P450 4A isozymes.

The physiological relevance of HNE and its different metabolites is not yet fully understood. This compound is known to have an important chemical reactivity towards thiols, (4, 5), amino groups of aminoacids (6–8), or nucleic acids in vitro (9, 10). In vivo, this compound was shown to be cytotoxic when given at a concentration that could be reached during oxidative stress (36). However, in vivo, at physiological concentrations that have shown to be in the range of 0.1 to 1.4 μm (37–39), the highly reactive aldehydic group seems to be very rapidly reduced to give rise to alcohol (DHN) or oxidized in acid (HNA) derivatives. These primary metabolites are supposed to be conjugated to glutathione (GSH) in a second step as evidenced by in vitro experiments (12, 13), as HNE-MA derivatives were not shown to be substrates for cytosolic alcohol and aldehyde dehydrogenase (11). Besides, HNE induces the in vitro transcription and expression of aldose reductase which limits its concentration in cells (40). These metabolization steps

considerably reduce the reactivity of the parent compound and increase its elimination. Extensive metabolization of HNA by P450 4A isoenzymes appears as another way to increase the water-solubility of this compound. In mice, this metabolization could perceptibly prevent any further conjugation to GSH as no 9-OH-HNA conjugated to mercapturic acid (MA) appears to be significantly increased after fibrate treatment. However, the persistence of the unsaturation in position 2 could confer a relative chemical reactivity to this hydroxylated HNA metabolite towards thiols. In fact, in the rat, the 9-OH-HNA-MA conjugate was found in urine (35), suggesting that 9-OH-HNA could be virtually considered as a substrate of glutathione S-transferases. This species difference between rats and mice in terms of ω-hydroxylated metabolites conjugated or not to N-acetylcysteine remains to be clarified.

In connection with the latter point, and despite this intensive metabolization into more easily excretable compounds, HNE was recently described as a key mediator of neuronal apoptosis induced by oxidative stress (3). These results indicate that this compound could be not only a terminal deleterious lipoperoxidation product but a fully bioactive molecule, particularly in stressful conditions, when cellular defense mechanisms against oxidative injury are overwhelmed, as anticipated previously (36). The involvement of P450 4A isoenzymes in neural tissue metabolism was reported for P450 4A2 and 4A3 with PCR amplification of RNA transcripts, even through the presence of this monoxygenase family in brain is far less important than in liver (41, 42). Investigation of the contribution of these enzymes to the basal oxidative metabolism of HNE in neural tissues could be of interest. Considering a putative biological implication of HNE or its derivatives, our results substantiate the key role of PPARα and its endogenous activation in establishing a basal level of expression of enzymatically relevant proteins as the cytochrome P450 4A isozymes. In fact, the relative concentrations of 4-hydroxy-2-nonene-1,9-dicarboxylic acid excreted in urine after [4-^{3}H]HNE administration can vary by a 7-fold ratio between a wild-type mouse and a PPARα-deficient mouse, from 0.55% to 0.08% of the total radioactivity analyzed, respectively. As HNE mediates apoptosis in neuronal cells (3), further investigations of the in vivo HNE-dependent susceptibility to apoptosis in PPARα-deficient mice could be of substantial interest in an engineered mouse line expressing reduced levels of endogenous cytochrome P450 4A.

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