Human Carboxylesterase 1A Plays a Predominant Role in Hydrolysis of the Anti-Dyslipidemia Agent Fenofibrate in Humans[S]

Hong-Xin Li, Meng-Ru Sun, Ya Zhang, Li-Lin Song, Feng Zhang, Yun-Qing Song,
Xu-Dong Hou, and Guang-Bo Ge

Shanghai Frontiers Science Center of TCM Chemical Biology, Institute of Interdisciplinary Integrative Medicine Research, Shanghai University of Traditional Chinese Medicine, Shanghai, China (H.-X.L., M.-R.S., Y.Z., L.-L.S., F.Z., Y.-Q.S., X.-D.H., G.-B.G.) and Liaoning Provincial Key Laboratory of Carbohydrates, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian, China (L.-L.S.)

ABSTRACT

Fenofibrate, a marketed peroxisome proliferator-activated receptor-α (PPARα) agonist, has been widely used for treating severe hypertriglyceridemia and mixed dyslipidemia. As a canonical prodrug, fenofibrate can be rapidly hydrolyzed to release the active metabolite (fenofibric acid) in vivo, but the crucial enzyme(s) responsible for fenofibrate hydrolysis and the related hydrolytic kinetics have not been well-investigated. This study aimed to assign the key organs and crucial enzymes involved in fenofibrate hydrolysis in humans, as well as reveal the impact of fenofibrate hydrolysis on its non-PPAR–mediated biologic activities. Our results demonstrated that fenofibrate could be rapidly hydrolyzed in the preparations from both human liver and lung to release fenofibric acid. Reaction phenotyping assays coupling with chemical inhibition assays showed that human carboxylesterase 1A (hCES1A) played a predominant role in fenofibrate hydrolysis in human liver and lung, while human carboxylesterase 2A (hCES2A) and human monoacylglycerol esterase (hMAGL) contributed to a very lesser extent. Kinetic analyses showed that fenofibrate could be rapidly hydrolyzed by hCES1A in human liver preparations, while the inherent clearance of hCES1A-catalyzed fenofibrate hydrolysis is much higher (>200-fold) than than that of hCES2A or hMAGL. Biologic assays demonstrated that both fenofibrate and fenofibric acid showed very closed Nrf2 agonist effects, but fenofibrate hydrolysis strongly weakens its inhibitory effects against both hCES2A and hNtoum. Collectively, our findings reveal that the liver is the major organ and hCES1A is the predominant enzyme-catalyzing fenofibrate hydrolysis in humans, while fenofibrate hydrolysis significantly reduces inhibitory effects of fenofibrate against serine hydrolases.

SIGNIFICANCE STATEMENT

Fenofibrate can be completely converted to fenofibric acid in humans and subsequently exert its pharmacological effects, but the hydrolytic pathways of fenofibrate in humans have not been well-investigated. This study reported that the liver was the predominant organ and human carboxylesterase 1A was the crucial enzyme involved in fenofibrate hydrolysis in humans.

Introduction

Fenofibrate, one of the most widely prescribed fibric acid derivatives, was approved for the treatment of hyperlipidemic by the US Food and Drug Administration in 1998 (Saurav et al., 2012). Following oral administration, fenofibrate can be quickly absorbed into the circulation system and then extensively hydrolyzed by esterases to release fenofibric acid (Guay, 1999, 2002; Filippatos and Milionis, 2008). It is generally recognized that fenofibrate exerts its anti-hyperlipidemic effect mainly through its active hydrolytic metabolite, fenofibric acid (Arakawa et al., 2005; Tsimihodimos et al., 2005; Zhu et al., 2010; Malátková et al., 2016). Fenofibric acid exerts a significant impact on the modulation of lipoprotein and fatty acid metabolism through its selective targeting of peroxisome proliferator-activated receptor-α(PPAR-α), leading to substantial reductions in total plasma cholesterol, low-density lipoprotein cholesterol, triglycerides, and very low-density lipoprotein levels. Additionally, it elicits an elevation in serum high-density lipoprotein cholesterol as well as lipoprotein AI and AII concentrations (Saurav et al., 2012; Malátková et al., 2016). Due to its excellent tolerability and high safety profiles, fenofibrate has been extensively used for the treatment of dyslipidemia all over the world in the past few decades, whether used alone or in combination with statins (Guay, 2002; McKeage and Keating, 2011; Saurav et al., 2012). In addition to the PPAR-α agonist effect, recent studies have reported that fenofibrate shows a wide range of biologic activities, from anti-inflammatory effect to the inhibitory effects against a variety of enzymes (Gong et al., 2016; Dai et al., 2017; Prasad et al., 2018; Li et al., 2021; Chen et al., 2022; Spartalis et al., 2022).

Although fenofibrate has been used clinically for several decades, its hydrolytic pathways in humans have not been well investigated yet.

ABBREVIATIONS: BNPP, bis-(p-nitrophenyl) phosphate; DME, D-luciferin methyl ester; GA, galanthamine; hAchE, human acetylcholinesterase; hBchE, human butyrylcholinesterase; hCES1A, human carboxylesterase 1A; hCES2A, human carboxylesterase 2A; HIMs, human intestine microsomes; HKM, human kidney microsomes; HLMs, human liver microsomes; HLuMs, human lung microsomes; hMAGL, human monoacylglycerol lipase; hNotum, human Notum; HPLC, high-performance liquid chromatography; hSA, human serum albumin; IC_{50}, half maximal inhibition concentration; LC, liquid chromatography; LPA, loperamide; PPAR-α, peroxisome proliferator activated receptor-alpha; SFN, sulforaphane.
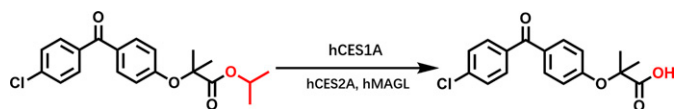
Fig. 1. The hydrolytic pathways of fenofibrate in human liver preparations and human lung preparations.

Following oral administration, fenofibrate can be rapidly hydrolyzed in the system to release fenofibric acid, which then enhances the PPAR-α signaling pathway to exert its major pharmacologic effects in vivo (Chapman, 1987; Adkins and Faulds, 1997; Miller and Spence, 1998; Streel et al., 2000; Ramjattan et al., 2002; Filippatos and Milionis, 2008; Fukami et al., 2015). Previous studies have indicated that the key enzymes involved in this process as well as the kinetics of fenofibrate hydrolysis are still poorly understood despite evidence of its release as fenofibric acid in vivo (Chapman, 1987; Kraja et al., 2010; Filippatos, 2012). It is well known that the hydrolytic metabolism may strongly affect the in vivo pharmacological effects of ester-bearing agents including prodrugs (Walther et al., 2017; Wind et al., 2019; Li et al., 2022). Although fenofibric acid has been found to have a PPAR-α agonist effect and an anti-inflammatory effect (Willson et al., 2000; Seber et al., 2006; Prasad et al., 2018; Spartalis et al., 2022; Jin et al., 2023), the impact of fenofibrate hydrolysis on its non-PPAR effects (such as Nrf2 agonist and enzyme inhibition) is poorly understood. Thus, it is imperative to ascertain the key hydrolases and primary metabolic organs involved in fenofibrate hydrolysis in humans and evaluate its impact on its biologic activities (Schelleman et al., 2014; Lee et al., 2020; Pristner and Warth, 2020; Bettonte et al., 2022).

This study aimed to address two key issues related to fenofibrate hydrolysis: (1) to identify the major organs and enzymes responsible for its hydrolysis in humans and (2) to investigate the impact of fenofibrate hydrolysis on non-PPAR effects (such as Nrf2 agonist effects and enzyme inhibition activities). To achieve these goals, the hydrolytic metabolite of fenofibrate in human plasma and tissue preparations was carefully characterized, while a suite of assays (including reaction phenotyping assays, chemical inhibition assays and hydrolytic kinetics assays) were conducted to identify the key contributing enzymes responsible for fenofibrate hydrolysis. Furthermore, the biologic activities of fenofibrate and its hydrolytic metabolite (fenofibric acid) were tested to reveal the impact of fenofibrate hydrolysis on its non-PPAR effects.

Materials and Methods

Materials and Reagents. Fenofibrate, sulforaphane, fenofibric acid and vildagliptin were provided by Meilun Biolo gy and Technology Co., Ltd. (Dalian, China). EDTA and galanthamine (GA) were produced by J&K Chemical Co., Ltd (Beijing, China). Loperamide (LPA) and bis(p-nitrophenyl) phosphate (BNPP) were purchased from TCI Development Co., Ltd (Shanghai, China). Luciferin detection reagent was provided by Promega Biotech (Madison, WI). Steady-Lumi II Firefly Luciferase Assay Kit was offered by Beyotime (Shanghai, China). High-performance liquid chromatography (HPLC)-grade methanol, acetonitrile and formic acid were provided by Sigma (St. Louis, MO).

Enzymes and Tissue Preparations. The human lung microsomes (HLuMs; lot no. X040032), human kidney microsomes (HKMs; lot no. X03801), human plasma, HS9 (human liver S9 fractions), human live cytosol, and 16 individual-donor HLM were provided by Research Institute for Liver Diseases (Shanghai,
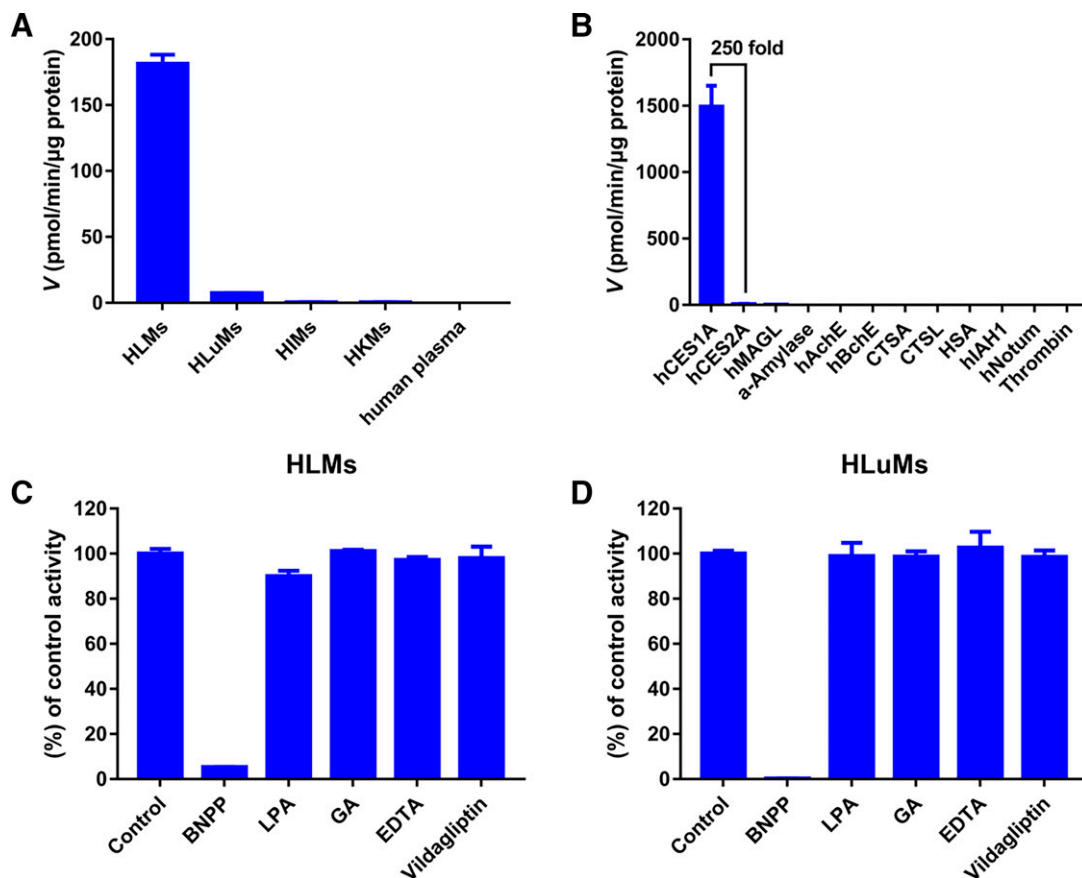

Fig. 2. (A) Reaction phenotyping assays of fenofibrate hydrolysis (10 μM) in the microsomes from human tissues (liver, lung, intestine, and kidney; the final concentration used was 50 μg/ml) and human plasma (25% dilution) for 30 minutes at 37°C. (B) Reaction phenotyping assays of fenofibrate hydrolysis (10 μM) using various human hydrolases (5 μg/ml, except that of thrombin was 5 NIH/ml) for 30 minute at 37°C. (C and D) Inhibitory effects of the esterase inhibitors toward fenofibrate hydrolysis in HLMs and HLuMs. All assays were conducted in triplicate, and the data are expressed as mean ± S.D.
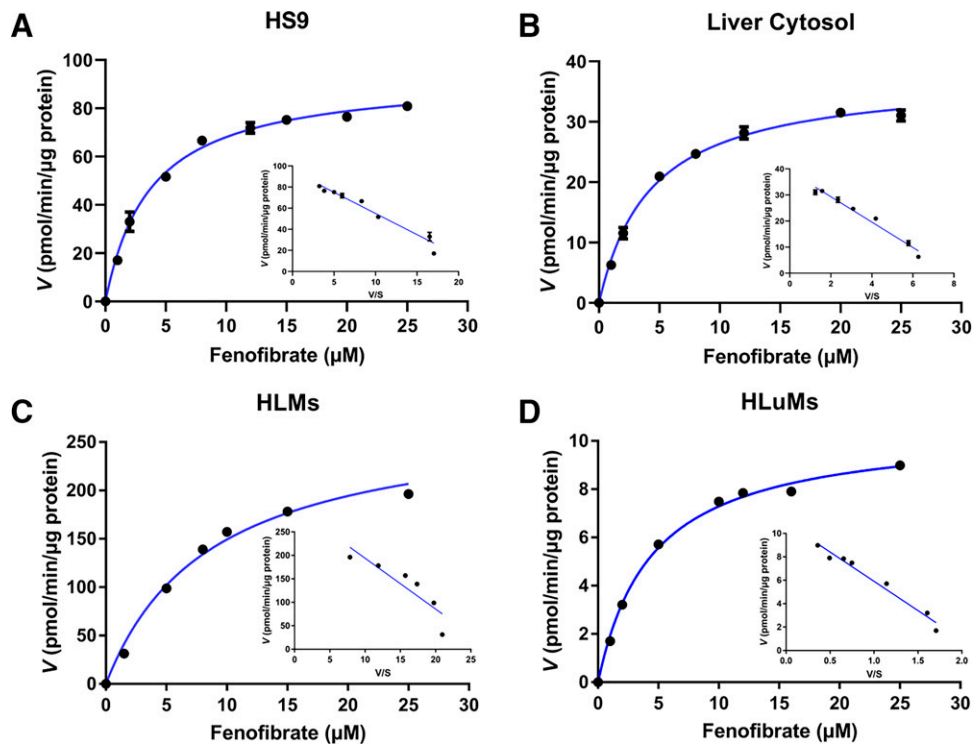
Fig. 3. Michaelis−Menten kinetic plots and the corresponding Eadie–Hoftsee plots (as the insert) of fenofibrate hydrolysis in HS9 (A), live cytosol (B), HLMs (C), and HLuMs (D). All assays were conducted in triplicate, while the data were expressed as mean ± S.D.

China). Pooled human liver microsomes HLMs from 50 donors (HLMs; lot no. 2010065) and pooled human intestine microsomes (HIMs; lot no. 1410066) from 6 donors were provided by Sekisui Xenotech (USA). Human acetylcholinesterase (hAchE; lot no. C1682), human butyrylcholinesterase (hBchE; lot no. SLCG5158), α-Amylase and human serum albumin (lot no. SLCC9268) were provided by Sigma-Aldrich (St. Louis, MO). Human monoacylglycerol lipase (hMAGL; lot no. 10007812) was purchased from Cayman Chemicals (USA) and human cathepsin A (lot no. FXY0619021) was purchased from R&D Systems Co., Ltd (USA). Human cathepsin L (lot no. C401) was purchased from Novoprotein (Shanghai, China). Recombinant human carboxylesterase 1A (hCES1A), human Notum (hNotum), human carboxylesterase 2A (hCES2A), and human isoamyl acetate-hydrolyzing esterase 1 were expressed and purified according to previously described methods (Fukuda et al., 2000). All biologic reagents were stored at −80°C.

Quantitative Analysis of Fenofibrate and Its Hydrolytic Metabolite(s) by HPLC-UV. An HPLC-UV system, consisting of a DGU-405 degassing unit, an SPD-M40 photo diode array detection, a SIL-40CXR autosampler, an LC-40BXR pump, a CTO-40S column oven, a CBM-40 system controller (Kyoto, Japan), and a C18 column (5 μm, 150 mm × 2.1 mm; Shimadzu, Kyoto, Japan), was applied to isolate and quantify fenofibrate and the hydrolytic metabolite. The mobile phases consisted of 0.1% formic acid in water (A) and acetonitrile (B), with the following elution gradients: 0 to 1.5 minutes, 60% B; 1.5 to 4 minutes, 60% to 95% B; 4 to 5.5 minutes, 95% B; and 5.5 to 6 minutes, 95% to 60% B;

after that, the solvent B was adjusted to its starting concentration of 60%. The flow rate was 0.4 ml/min and the column temperature was set at 40°C. The detection wavelength of fenofibrate and its hydrolytic metabolite(s) was set at 280 nm (the maximum absorbance wavelength of two analytes in UV).

Phenotyping Assays Using Human Hydrolases. The hydrolytic rates of fenofibrate in various recombinant enzymes (hCES1A, hCES2A, hMAGL, α-Amylase, hBchE, hAchE, human cathepsin A, human cathepsin L, thrombin, human serum albumin, hNotum, human isoamyl acetate-hydrolyzing esterase 1) were evaluated under identical conditions (pH 7.4 at 37°C), utilizing the same final substrate concentration (10 μM). Each enzyme was set at a final concentration of 5 μg/ml except for thrombin (5 NIH/ml, final concentration). After 0.5-hour incubation at 37°C, the reaction was quenched by adding ice-cold acetonitrile. Followed by centrifugation (20,000 × g for 30 minutes) at 4°C, the supernate was obtained for liquid chromatography (LC)-UV analysis.

Chemical Inhibition Assays. The involvement of esterases in the hydrolysis of fenofibrate was also investigated by employing various inhibitors targeting specific human hydrolases, including BNPP (a specific inhibitor of CES), LPA (a selective inhibitor for hCES2A), GA (a selective hAchE inhibitor), EDTA (a selective PONs inhibitor), and vildagliptin (a selective inhibitor for human dipeptidyl peptidase IV) (Warner et al., 1992; Quinney et al., 2005; Lauster et al., 2007; He et al., 2009; Zou et al., 2016, 2018; Buckler et al., 2017). The reaction system (200 μl) contained PBS (pH 7.4), HLMs or HLuMs, inhibitors, and fenofibrate (10 μM). The incubation was carried out at 37°C for 0.5 hours and terminated by adding 200 μl acetonitrile. After centrifugation (20,000 × g for 30 minutes) at 4°C, the supernatants were collected for LC-UV analysis. Fenofibrate and its hydrolytic metabolite were detected by LC-UV at 280 nm.

Fenofibrate Hydrolytic Kinetics. The enzymatic kinetics of fenofibrate hydrolysis were analyzed in various tissue preparations (HS9, live cytosol, HLMs, HLuMs) and human recombinant enzymes (hCES1A, hCES2A, hMAGL) through incubation with a range of concentrations of fenofibrate at 37°C in triplicate. After 0.5-hour incubation at 37°C, the reaction was quenched by adding ice-cold acetonitrile. Followed by centrifugation (20,000 × g for 30 minutes) at 4°C, the supernate was obtained for LC-UV analysis. The kinetic constants were calculated by fitting the data to the Michaelis–Menten equation using nonlinear least-squares regression as previously reported (Zhang et al., 2022).

TABLE 1

Kinetic parameters of fenofibrate hydrolysis in HLMs, HLuMs, hCES1A, hCES2A, and hMAGL

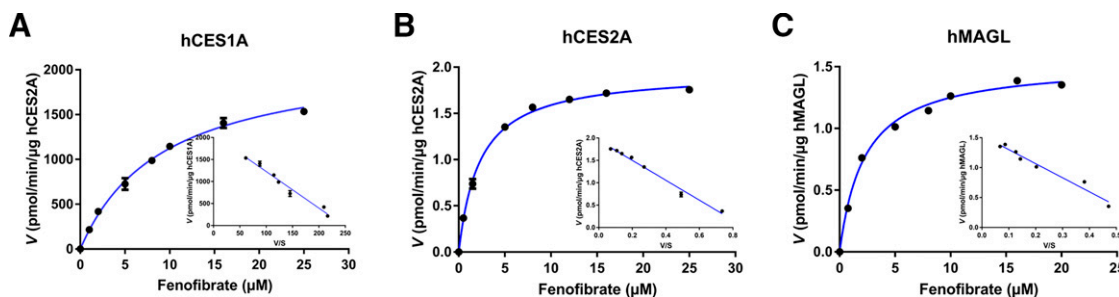| Enzyme source	K_m (μM)	V_{max} (pmol/min/μg protein)	CL_{int} (μl/min/μg protein)
HS9	3.75 ± 0.27	93.56 ± 1.93	25.0
Live cytosol	4.27 ± 0.28	37.6 ± 0.75	8.81
HLMs	8.63 ± 1.07	278.10 ± 15.05	32.24
HLuMs	4.69 ± 0.22	10.83 ± 0.15	2.31
hCES1A	9.27 ± 0.92	2165.00 ± 94.16	233.55
hCES2A	2.29 ± 0.11	1.95 ± 0.022	0.85
hMAGL	2.25 ± 0.19	1.53 ± 0.031	0.68

Fig. 4. Michaelis−Menten kinetic plots and the corresponding Eadie–Hoftsee plots (as the insert) of fenofibrate hydrolysis in hCES1A (A), hCES2A (B), and hMAGL (C). All assays were conducted in triplicate, and the data are expressed as mean ± S.D.

Interindividual Variability in CES1A Activity and Correlation Study. Based on the previously mentioned procedure, fenofibrate (10 μM) was incubated with a panel of 16 individual at 37°C for 0.5 hours. The reaction was terminated by adding 200 μl ice-cold acetonitrile. Followed by centrifugation (20,000 × g for 30 minutes) at 4°C, the supernate was obtained for LC-UV analysis. To assess the hCES1A activities of the 16 individual HLMs, D-luciferin methyl ester (DME), a specific substrate of hCES1A was used as previously reported (Devineni et al., 2015; Wang et al., 2016, 2018). Hydrolytic rates of fenofibrate and DME in each HLM sample were determined and compared with validate the reliability of using fenofibrate as an indicator for monitoring hCES1A activity in HLMs. Linear regression analysis was performed to determine the correlation between the hydrolytic rates of fenofibrate and DME in each HLM sample.

Nrf2 Agonist Activities of Fenofibrate and Fenofibric Acid. The Nrf2 stable transfection HEK293 cells (Genomeditech, Shanghai, China) were grown as a monolayer in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and incubated at 37°C with 5% CO$_2$.

The cells were seeded at a density of 2×10^4 cells per well in a volume of 100 μl in a 96-well plate and incubated for 24 hours. After that, fenofibrate, fenofibric acid, and the positive control (sulforaphane) were added and incubated for another 24 hours. Subsequently, the Nrf2 agonist activity was assessed by measuring luciferase activity (Li et al., 2023).

Results

Characterization of the Hydrolytic Metabolite(s) of Fenofibrate. First, the hydrolytic metabolite(s) of fenofibrate in human plasma and different tissue preparations (such as HLMs, HLuMs, HIMs, and HKMs) were analyzed using both HPLC-UV and UHPLC-Q-TOF-MS/MS. Fenofibrate underwent rapid hydrolysis to yield a stable polar product in both HLMs and HLuMs (Figs. 1 and 2A; Supplemental Fig. 1). By contrast, a little amount of fenofibrate was found to be hydrolyzed in HIMs and HKMs, while no metabolite was detected in human plasma. The molecular ion [(M+H)$^+$] of this polar metabolite in positive ion mode was m/z 319.0738 Da, which is 42 Da lower than the prototype fenofibrate [m/z 361.1208 Da, (M+H)$^+$], indicating that the polar metabolite resulted from ester bond cleavage of fenofibrate. The fragmentation ions and retention time of this hydrolytic product were identical to fenofibric acid (Supplemental Figs. 2 and 3), suggesting rapid hydrolysis of fenofibrate in human liver and lung tissue preparations. This stimulated further investigation into the key enzymes involved in fenofibrate hydrolysis within these organs.

Identifying the Key Enzymes Involved in Fenofibrate Hydrolysis in Humans. To elucidate the key enzymes involved in the hydrolysis of fenofibrate in humans, hydrolytic reaction phenotyping assays and chemical inhibition assays were performed. First, the formation rates of fenofibric acid (the hydrolytic metabolite of fenofibrate) were determined in various hydrolases distributed in humans. As depicted in Fig. 2B, hCES1A exhibited a faster rate of hydrolysis toward fenofibrate, while hCES2A and hMAGL also catalyzed this reaction. Subsequently, a panel of specific inhibitors targeting human hydrolases was employed to identify the contributions made by different enzymes to fenofibrate hydrolysis in HLMs and HLuMs. As shown in Fig. 2C, BNPP strongly inhibited the hydrolytic metabolism of fenofibrate in HLMs, whereas LPA only slightly suppressed its hydrolysis. Conversely, GA, EDTA, and vildagliptin exhibit negligible impacts on the hydrolysis of fenofibrate in HLMs. Similarly, BNPP almost completely inhibited
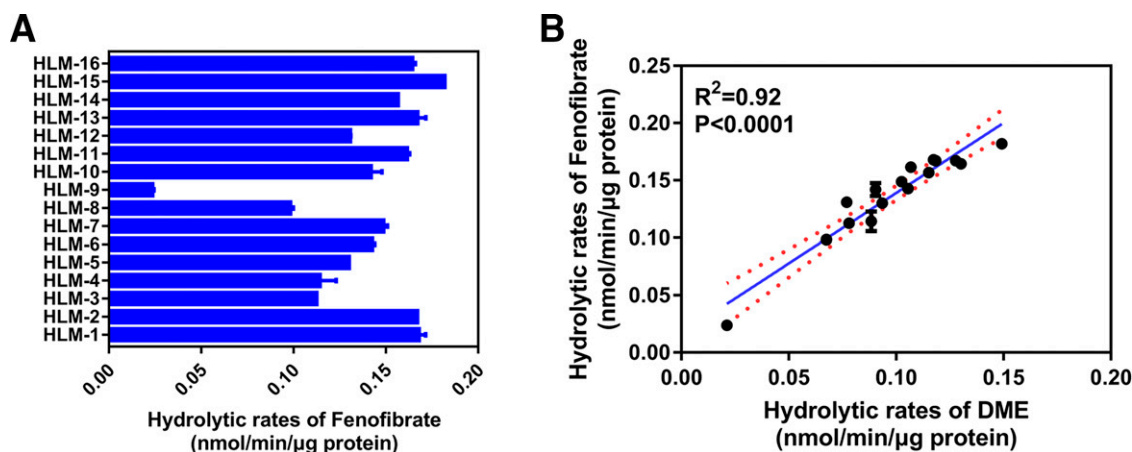

Fig. 5. (A) The hydrolytic rates of fenofibrate (10 μM) in human liver specimens from 16 individual donors (1 μg/ml). (B) Correlation analysis between the hydrolytic rates of fenofibrate and the hydrolytic rates of DME in individual human liver specimens ($n = 16$). All assays were conducted in triplicate, and the data are expressed as mean ± S.D.
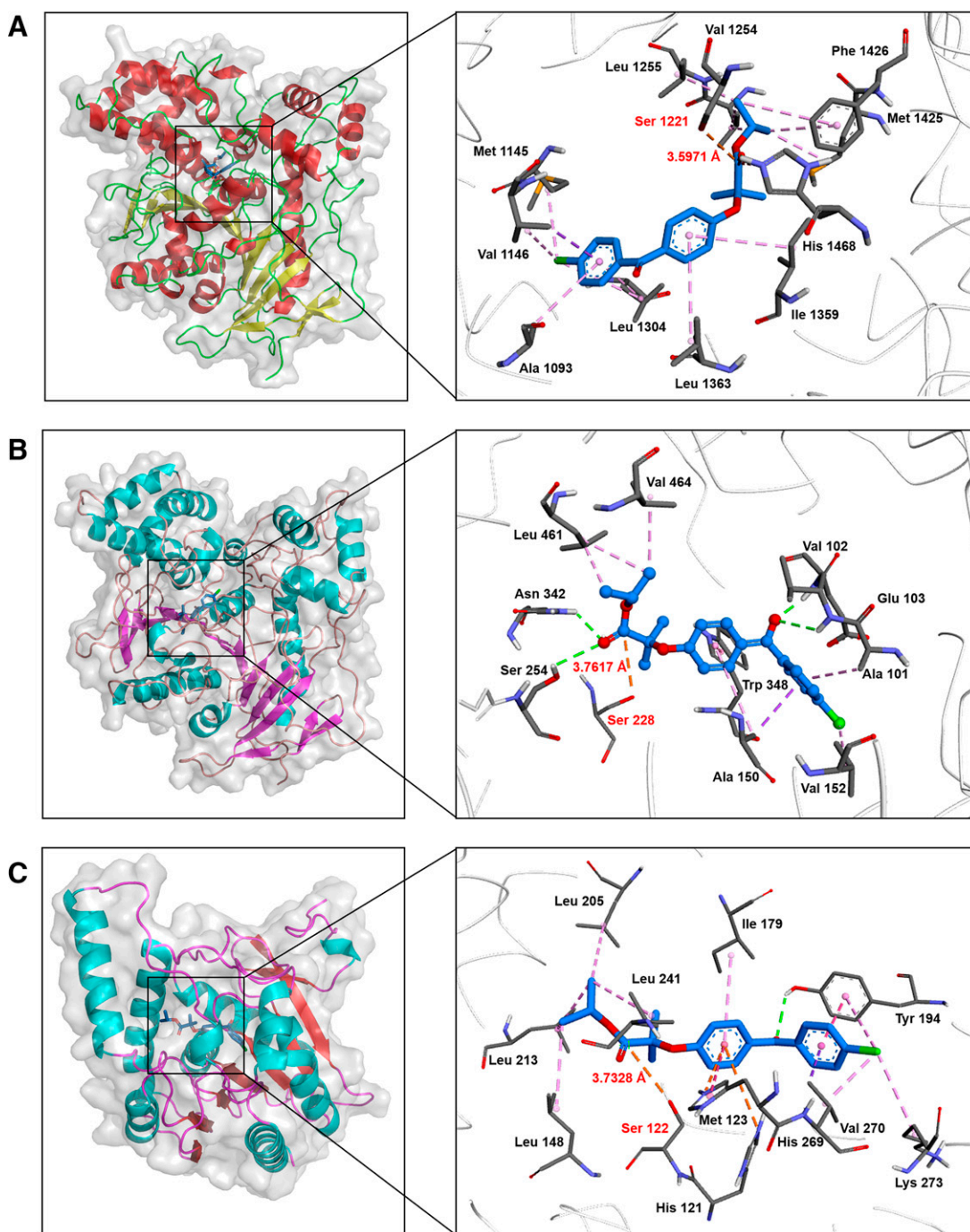
Fig. 6. The binding modes and enzyme-substrate interactions of fenofibrate (blue) in hCES1A (A), hCES2A (B), and hMAGL (C).

fenofibrate hydrolysis in HLuMs (Fig. 2D), whereas LPA, GA, EDTA, and vildagliptin hardly blocked this reaction. These observations clearly suggest that hCES1A plays a crucial role in fenofibrate hydrolysis in both HLMs and HLuMs, while hCES2A and hMAGL have only minor contributions to this process in the human liver.

Fenofibrate Hydrolytic Kinetics in Various Enzyme Sources. Next, the kinetic parameters (K_m and V_{max}) of fenofibrate hydrolysis were meticulously characterized in various enzyme sources, including tissue preparations (HS9, live cytosol, HLMs, HLuMs), as well as recombinant enzymes (hCES1A, hCES2A, and hMAGL). As shown in

Fig. 3 and Table 1, fenofibrate hydrolysis in different human tissue preparations followed classic Michaelis–Menten kinetics. The K_m values of fenofibrate hydrolysis in HS9 and live cytosol were 3.75 μM and 4.27 μM, respectively, while the V_{max} values of fenofibrate hydrolysis in HS9 and live cytosol were determined as 93.56 pmol/min/g protein and 37.60 pmol/min/g protein, respectively. The K_m values for fenofibrate hydrolysis in HLMs and HLuMs were determined as 8.63 μM and 4.69 μM, respectively, while the V_{max} values of fenofibrate hydrolysis in HLMs and HLuMs were 278.10 pmol/min/g protein and 10.83 pmol/min/g protein, respectively. As a result, the inherent clearance (C_{Lint}) values of fenofibrate hydrolysis in HS9, live cytosol, HLMs, and

TABLE 2

Docking simulations of fenofibrate into the catalytic cavity hCES1A, hCES2A, hMAGL, hAchE, and hBchE

Enzyme	Crystal structure	Affinity (kcal/mol)	Distance (Å)
hCES1A	2dr0	−9.211	3.60
hCES2A	homology modeling	−8.595	3.79
hMAGL	3pe6	−8.494	3.73
hAchE	4ey7	−7.846	10.20
hBchE	1p0i	−7.771	11.20

HLuMs were calculated as 25.3 μl/min/μg protein, 8.81 μl/min/μg protein, 32.24 μl/min/μg protein, and 2.31 μl/min/μg protein, respectively. These results unequivocally prove that the liver is the primary organ responsible for fenofibrate hydrolysis in humans.

As depicted in Fig. 4, Supplemental Fig. 4, and Table 1, fenofibrate hydrolysis in hCES1A, hCES2A, and hMAGL also followed classic Michaelis–Menten kinetics. The K_m values of fenofibrate hydrolysis in hCES1A, hCES2A, and hMAGL were determined as 9.27 μM, 2.29 μM, and 2.25 μM, respectively. The V_{max} values of fenofibrate hydrolysis were 2165 pmol/min/μg protein, 1.95 pmol/min/μg protein, and 1.53 pmol/min/μg protein in hCES1A, hCES2A, and hMAGL, respectively. Consequently, the C_{Lint} value of fenofibrate hydrolysis in hCES1A was very high (233.55 μl/min/μg protein), while the C_{Lint} values of fenofibrate hydrolysis in hCES2A and hMAGL were determined to be as low as 0.85 μl/min/μg protein and 0.68 μl/min/μg protein, respectively. It is obvious from Table 1 that the inherent clearance of hCES1A-catalyzed fenofibrate hydrolysis is 275-fold higher than that of hCES2A and 338-fold higher than that of hMAGL. These results clearly demonstrate that hCES1A contributes significantly to the hydrolytic metabolism of fenofibrate in humans, while hCES2A and hMAGL contribute this reaction to a much lesser extent.

Fenofibrate Hydrolytic Rates in HLMs Agreed Well with hCES1A Activities. Next, the metabolic rates of fenofibrate in 16 individual HLMs samples were assessed. As shown in Fig. 5A, HLMs samples from 16 individual donors showed different hydrolytic rates toward fenofibrate with approximately 10-fold variation in the hydrolytic rate. Notably, the metabolic rates of fenofibrate in 16 HLM samples showed a substantial association with the hydrolytic rates of DME (Fig. 5B). This finding further confirms that hCES1A plays a crucial role in fenofibrate hydrolysis and suggests that it affects the activation and in vivo therapeutic efficacy of fenofibrate in individuals.

Docking Simulations. Docking simulations were performed to investigate the interaction mechanisms between fenofibrate and hCES1A, hCES2A, hMAGL, hAchE, and hBchE. The results in Fig. 6 and Table 2 show that fenofibrate exhibited favorable docking into the catalytic

cavities of hCES1A, hCES2A, and hMAGL with low predicted binding affinities of −9.211 kcal/mol, −8.595 kcal/mol, and −8.494 kcal/mol, respectively. These results agree well with the high binding affinities of fenofibrate toward these three enzymes (Table 2). It is also clear from Fig. 6 that the distances between the carbonyl carbon of the isopropyl ester on fenofibrate and the Ser in the catalytic triad of hCES1A, hCES2A, and hMAGL were 3.60 Å, 3.79 Å, and 3.73 Å, respectively. These observations show that fenofibrate is a favorable substrate for hCES1A, while it can also undergo hydrolysis by both hCES2A and hMAGL.

It was also found that fenofibrate could be docked into hAchE and hBchE, but the distance between the carbonyl carbon of the methyl propanoic acid isopropyl ester on fenofibrate and the O^β atom of Ser in the catalytic triad of either hAchE or hBchE was significantly greater than the catalytic distance (<5.5 Å), indicating that fenofibrate was difficult to be hydrolyzed in either hBchE or hAchE. These observations align well with experimental results, which demonstrate that two carboxylesterases (hCES1A and hCES2A) and hMAGL are capable of hydrolyzing fenofibrate, while neither hBchE nor hAchE can catalyze its hydrolysis.

Fenofibrate Hydrolysis Strongly Weakens Its Inhibitory Effects Against Two Serine Hydrolases. It has been reported that fenofibrate could inhibit some important serine hydrolases including hNotum and hCES2A. In these cases, we tested the inhibitory effects of both fenofibrate and fenofibric acid against both hCES2A and hNotum, while LPA and LP922056 were used as positive inhibitors. As illustrated in Figs. 7 and 8, fenofibrate could dose-dependently inhibit both hCES2A and hNotum, with the IC_{50} values of 0.50 μM and 18.4 μM, respectively. By contrast, fenofibric acid exhibited weak inhibitory effects with IC_{50} values greater than 100 μM for both serine hydrolases (Supplemental Fig. 5). These results clearly suggest that fenofibrate hydrolysis will significantly reduce the inhibitory effects of this agent on serine hydrolases.

Fenofibrate and Fenofibric Acid Showed Similar Nrf2 Agonist Effects. It has also been reported that fenofibrate can resist cellular oxidative stress and attenuate diabetic nephropathy via enhancing the Nrf2 signaling pathway (Liu et al., 2018; Cheng et al., 2020; Li et al., 2021). Herein, the agonist effects of both fenofibrate and fenofibric acid on Nrf2 were tested at cellular levels. As shown in Fig. 9, both fenofibrate and its hydrolytic metabolite fenofibric acid showed similar Nrf2 agonist effects at three different doses. These findings provide clear evidence that both fenofibrate and fenofibric acid exhibit similar Nrf2 agonist effects, indicating that the hydrolysis of fenofibrate does not impact its Nrf2 agonist effect in vivo.

Discussion

Fenofibrate is a marketed fibric acid ester derivative that has been used globally to treat dyslipidemia for several decades. Previous
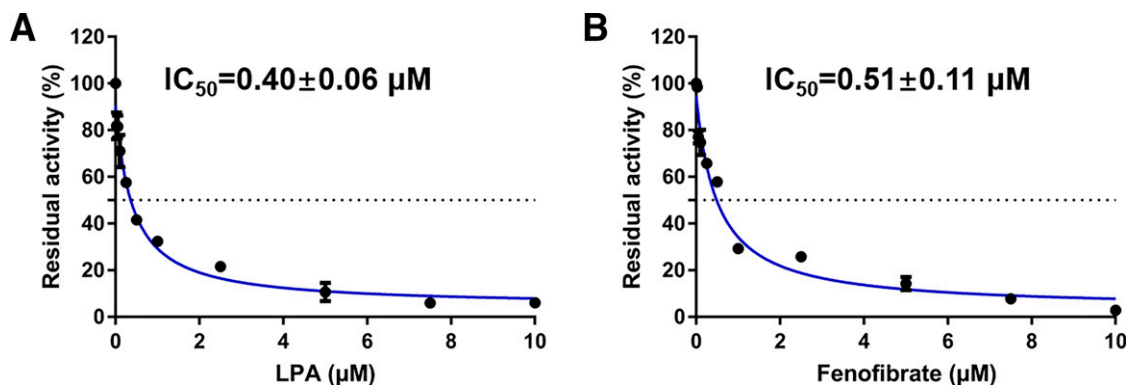

Fig. 7. (A) The dose-inhibition curves of LPA against hCES2A-catalyzed FD hydrolysis. (B) The dose-inhibition curves of fenofibrate against hCES2A-catalyzed FD hydrolysis. Data points were shown as mean ± SD ($n = 3$).
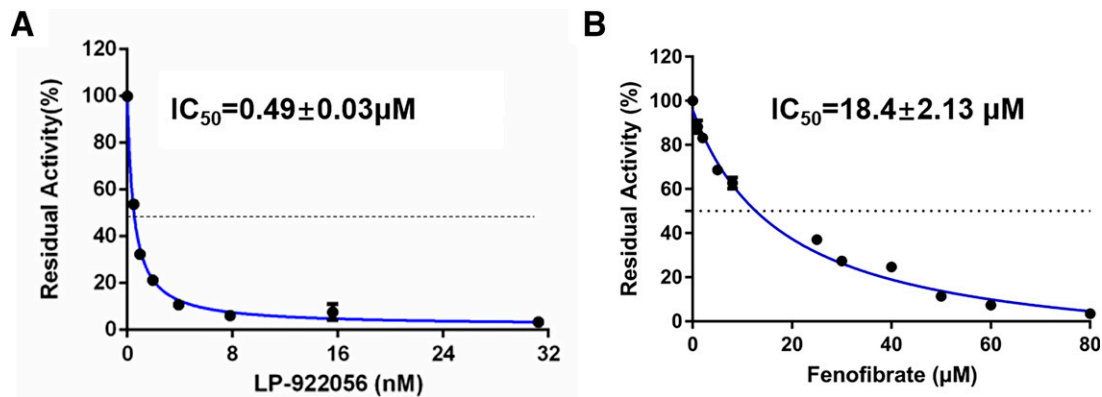
Fig. 8. (A) The dose-inhibition curves of LP-922056 against hNotum-catalyzed OPTS hydrolysis. (B) The dose-inhibition curves of fenofibrate against hNotum-catalyzed OPTS hydrolysis. Data points are shown as mean ± S.D. ($n = 3$).

investigations have shown that rapid hydrolysis of this agent to release fenofibric acid in humans, but the hydrolytic pathways and involved human enzymes have not been revealed yet. Herein, this research provides a detailed description of the hydrolytic pathway(s) of fenofibrate and key enzymes involved in its hydrolysis in humans for the first time. Our results clearly showed that fenofibrate could be readily hydrolyzed to release fenofibric acid in human liver and lung tissue preparations. In view of the fact that the weight of the human liver is larger than that of the lung and that the yield of microsomal protein obtained from the liver is 10- to 20-fold greater than that from the lung (Pacifici et al., 1988; Mutch et al., 2007; Doerksen et al., 2021), it is conceivable that the liver is the most important metabolic organ responsible for fenofibrate hydrolysis in humans. Meanwhile, our findings also suggested that hCES1A, hCES2A, and hMAGL are involved in the hydrolysis of fenofibrate. Considering that hCES1A is a highly abundant esterase distributed in the liver, the protein level of hCES1A in this organ is about 9.6-fold greater than that of hCES2A (Sato et al., 2012; Boberg et al., 2017). Meanwhile, as listed in Table 1, the inherent clearance of fenofibrate in hCES1A is much higher than that in hCES2A (~275-fold) and in hMAGL (~343-fold). These findings well-explain why LPA (a specific hCES2A inhibitor) shows a very weak inhibitory effect on fenofibrate hydrolysis in both HLMs and HLuMs and also suggest that hCES1A is a main enzyme involved in fenofibrate hydrolysis in both HLMs and HLuMs.

One of the most significant hydrolases in the human liver, hCES1A is primarily distributed in the endoplasmic reticulum of hepatocytes and is involved in the hydrolytic metabolism of a wide range of medications that contain ester or amide bond(s) (Mentlein and Heymann, 1984;

Redinbo and Potter, 2005; Landowski et al., 2006; Jin, et al., 2022). Previous studies have demonstrated that hCES1A prefers to hydrolyze the esters with a small alcohol group and a large carboxyl group, whereas hCES2A tends to hydrolyze the esters with a large carboxyl group and a small alcohol group (Satoh and Hosokawa, 2006; Hosokawa et al., 2007; Sanghani et al., 2009; Lian et al., 2018). Structurally, fenofibrate is a carboxylic ester bearing a relatively large carboxyl group and a small alcohol group, which aligns with the substrate specificity exhibited by hCES1A. It is well-known that hCES1A predominantly localizes in the human liver, whereas hCES2A primarily resides in the small intestine. In this study, our results unequivocally demonstrated that the liver served as the primary metabolic organ and that hCES1A was the principal enzyme responsible for hydrolysis of hCES1A in humans. Our findings agree well with the tissue distribution and substrate specificity of hCES1A, suggesting that hCES1A-catalyzed fenofibrate hydrolysis is the predominant metabolic pathway of fenofibrate in humans.

Many previous studies have demonstrated that fenofibrate exerts its anti-dyslipidemia effect mainly by activating PPAR-α. However, fenofibrate could be rapidly hydrolyzed to release its hydrolytic metabolite in vivo, and this conversion is complete (Chapman, 1987; Guay, 1999; McKeage and Keating, 2011). Several studies have reported that fenofibric acid ($EC_{50} = 18$–20 μM) showed a more potent agonist effect on PPAR-α when compared with fenofibrate ($EC_{50} = \sim30$ μM) (Willson et al., 2000; Kuwabara et al., 2004; Dietz et al., 2012; Honda et al., 2022). These data suggested that fenofibric acid rather than fenofibrate was the key bioactive substance for the treatment of dyslipidemia effect in vivo. It should also be noted that fenofibrate is not an ideal prodrug for treating dyslipidemia owing to the poor water solubility and low
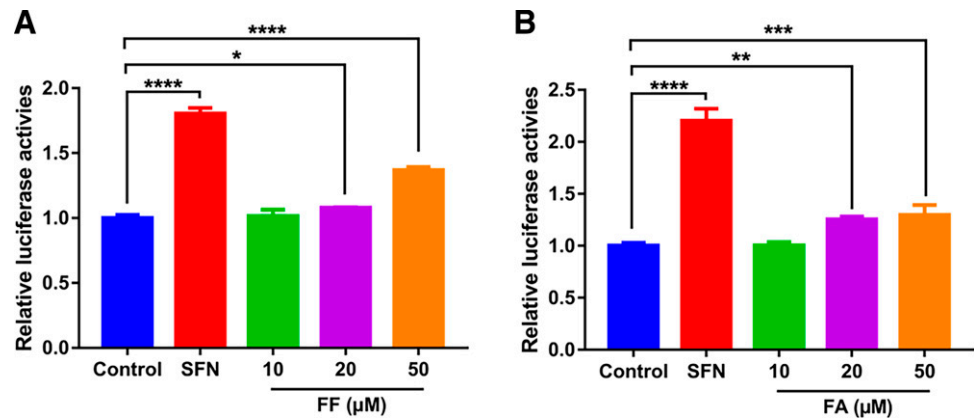
Fig. 9. The agonist effects of fenofibrate and fenofibric acid on Nrf2. (A) The cells were treated with the Nrf2 agonist sulforaphane (10 μM) or fenofibrate or fenofibric acid (12.5, 25,50 μM) for 24 hours. Compared to the control group (DMSO), ****$P < 0.0001$, ***$P < 0.001$, **$P < 0.01$, and *$P < 0.1$. FA, fenofibric acid; FF, fenofibrate.

oral availability in humans (McKeage and Keating, 2011; Saurav et al., 2012). In these cases, more water-soluble fenofibrate derivatives, including water-soluble prodrugs should be designed and developed, aiming to find novel and ideal fenofibrate prodrugs for translational applications. The ideal oral-administrated fenofibrate prodrugs should be well absorbed into the circulatory system, while they can be slowly hydrolyzed by esterases in the human body and then release the active hydrolytic product (fenofibric acid). In view of the fact that the esters of fenofibric acid also displayed a potent PPAR-α agonist effect, more esters of fenofibric acid can be designed and synthesized by medicinal chemists to study the structure-activity (PPAR-α agonist effect) relationships of these derivatives. Meanwhile, the oral bioavailability and hydrolytic kinetics of the esters of fenofibric acids with a potent PPAR-α agonist effect should also be investigated.

The agonistic effects of fenofibrate and its metabolite fenofibric acid on PPAR-α have been reported previously (Willson et al., 2000; Kuwabara et al., 2004; Dietz et al., 2012; Honda et al., 2022). Meanwhile, some non-PPAR mediated biologic activities of fenofibrate and fenofibric acid have also been reported (Blanco-Rivero et al., 2007; Chen et al., 2007; Cheng et al., 2020; Li et al., 2021). Notably, it has been reported that fenofibrate could regulate the expression and activity levels of some important hydrolases, such as PON1 in HuH7 cells and MMP9 in macrophages (Ronsein et al., 2016). Fenofibrate could also downregulate MMP9 in macrophages, and its hydrolytic metabolite fenofibric acid also downregulated MMP9 and inhibited MMP9 enzymatic activity in macrophages (Shu et al., 2000; Rival et al., 2004; Delayre-Orthez et al., 2005). These results have prompted us to further explore the non-PPAR effects of both fenofibrate and its hydrolytic metabolite fenofibric acid in humans. In this study, our findings indicated that fenofibrate was a potent inhibitor of hCES2A (IC$_{50}$ = 0.51 μM) and acted as a moderate inhibitor of hNotum (IC$_{50}$ = 18.4 μM), but its hydrolytic metabolite fenofibric acid displayed very weak inhibitory effects on these two serine hydrolases (IC$_{50}$ > 100 μM). These findings clearly suggest that fenofibrate hydrolysis will strongly reduce its inhibitory effects on both hCES2A and hNotum in vivo.

In summary, our investigation identified the primary metabolic organs and important hydrolases involved in fenofibrate hydrolysis in humans and revealed the differential impact of fenofibrate hydrolysis on its biologic activities. According to our research, hCES1A (a predominant serine hydrolase distributed in human liver) was the crucial enzyme responsible for fenofibrate hydrolysis in humans, whereas hCES2A and hMAGL contributed slightly to this reaction. Further investigations suggested that fenofibrate hydrolysis would significantly reduce the inhibitory effects of this agent on two serine hydrolases (hNotum and hCES2A), while such biotransformation did not affect its Nrf2 agonist effect. All these findings offer a deeper understanding of the hydrolytic pathways of the anti-dyslipidemia agent fenofibrate in humans, which is also helpful for medicinal chemists to develop novel ester prodrugs of fenofibrate with desirable pharmacokinetic behaviors.

Data Availability

The authors declare that all the data supporting the findings presented in this study are contained within the paper or its Supplemental Material.

Authorship Contributions

Participated in research design: Li, Sun, F. Zhang, Y-Q. Song, Hou, Ge.
Conducted experiments: Li, Y. Zhang.
Contributed new reagents or analytic tools: L-L. Song.
Performed data analysis: Li, Y. Zhang, F. Zhang.
Wrote or contributed to the writing of the manuscript: Li, Ge.

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Address correspondence to: Guang-Bo Ge, Institute of Interdisciplinary Integrative, Medicine Research, Shanghai University of Traditional Chinese Medicine, Shanghai 201203, China. E-mail: geguangbo@shutcm.edu.cn