Increased Levels of Fatty Acids Contributed to Induction of Hepatic CYP3A4 Activity Induced by Diabetes — In Vitro Evidence From HepG2 Cell and Fa2N-4 Cell Lines

Nan Hu¹², Mengyue Hu¹,#, Ru Duan¹, Can Liu¹, Haifang Guo¹, Mian Zhang¹, Yunli Yu¹, Xinting Wang¹, Li Liu¹,*#, and Xiaodong Liu¹,*b

¹Key Laboratory of Drug Metabolism and Pharmacokinetics, China Pharmaceutical University, Nanjing 210000, Jiangsu, China
²Department of Clinical Pharmacy, The Third Affiliated Hospital of Soochow University, Changzhou 213000, Jiangsu, China

Received October 23, 2013; Accepted January 29, 2014

Abstract. Accumulating evidences have shown that diabetes upregulated the function and expression of CYP3A4, but the mechanism remained unclear. In this study, HepG2 cells were incubated with serum from diabetic rats induced by streptozotocin, and the activity of CYP3A4 was measured by substrate metabolism. Results showed that incubation with diabetic serum significantly induced CYP3A4 activity in HepG2 cells. To identify the specific factors contributing to the regulation, the abnormally altered components in diabetic serum, including glucose, insulin, cholesterol, and free fatty acids were screened. It was found that only fatty acids concentration-dependently up-regulated CYP3A4 activity, and the induction by fatty acids was further confirmed in Fa2N-4 cells. Data from western blotting and QT-PCR showed that induction of CYP3A4 activity was associated with up-regulation of CYP3A4 protein and mRNA levels. In addition, effects of pharmacological inhibitors on fatty acid–induced CYP3A4 activity were studied. The results indicated that the induction of CYP3A4 activity by oleic acid may be partly via AMPK-, PKC-, and NF-κB–dependent pathways, whereas that by palmitic acid was possibly associated with the PKC-dependent pathway. In conclusion, the increased levels of fatty acids may be one of the reasons leading to the elevated function and expression of CYP3A4 under diabetic conditions.

Keywords: diabetes, fatty acid, CYP3A4, HepG2, Fa2N-4

Introduction

Accumulated evidences have demonstrated that diabetes mellitus markedly alters the activity and expression of cytochromes P450 (CYP450) enzymes (1–4). In diabetic rats, the expressions of CYP1A1, 1A2, 2C12, 2E1, and 3A1/2 were apparently increased, while those of CYP2C11, 2C13, and 2A2 were suppressed (2–5). Our previous study also demonstrated that the expression and activity of hepatic CYP3A were up-regulated in diabetic rats induced by a high dose of streptozocin (STZ) (model of type 1 diabetes) (6) as well as diabetic rats induced by combination of high-fat diet followed by a low dose of STZ (model of type 2 diabetes) (7).

It is generally accepted that diabetes is associated with insulin deficiency or insulin resistance. Several reports have showed that insulin treatment may reverse alteration in drug-metabolizing enzyme activity and expression induced by diabetes (5, 8), inferring contribution of insulin to alteration in CYP450 activity and expression under diabetic conditions. Other factors such as testosterone, tetraiodothyronine (8), and ketone body (9) were reported to be involved in regulation of CYP450 activity in diabetes mellitus. However, the mechanism leading to the alteration in hepatic CYP3A under the diabetic
condition was not fully understood, although the diabetes-mediated up-regulation of CYP2E1 is considered to be attributed to the increased level of ketone bodies (10).

The aim of this study was firstly to investigate the effects of diabetes on CYP3A4 activity in HepG2 cell model using diabetic rat serum to mimic the diabetic situation and secondly, to clarify whether abnormal components in diabetic serum including high levels of plasma glucose, cholesterol, free fatty acids as well as low levels of insulin were involved in regulation of CYP3A4 activity in HepG2 cell lines. Some results were further verified using Fa2N-4 cell lines; finally, to document the underlying mechanisms contributing to the alteration in CYP3A4 activity via application of different pharmacological inhibitors on HepG2 cells.

Materials and Methods

Materials

Verapamil hydrochloride, ketoconazole, midazolam, and erythromycin were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). 1'-Hydroxymidazolam (1'-OH MDZ), norverapamil hydrochloride, insulin, palmitic acid (PA), oleic acid (OA), stearic acid (SA), linoleic acid (LA), and streptozotocin (STZ) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Cholesterol and rifampicin were purchased from Aladin Reagent (Shanghai Jingchun Chemical Co., Ltd., Shanghai, China). 1α, 25-Dihydroxyvitamin D3 (VD3) was purchased from Xinjing Pharmachem Co., Ltd. (Xi’an, China). Dubelco’s Modified Eagle Medium (DMEM), penicillin, streptomycin, and non-essential amino acids (NEAA) were obtained from Gibco (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from PAA (Pasching, Austria). Fatty acid free BSA was purchased from Qebio Science & Technologies Co., Ltd. (Shanghai, China). 2-Nitrophenylhydrazine hydrochloride (2-NPH·HCl) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (1-EDC·HCl) were purchased from J&K Chemical, Ltd. (Beijing, China). Cryopreserved Fa2N-4 cells, Multi-Function Enhancing (MFE) supporting medium and MFE plating medium were purchased from XenoTech LLC (Lenexa, KS, USA). All the other reagents were of analytical grade and were commercially available.

HepG2 cell and Fa2N-4 cell culture

HepG2 cells were obtained from the American Type Culture Collection and cultured in DMEM (25 mM glucose) supplemented with 100 mg/ml penicillin, 100 U/ml streptomycin, 2.5 mM l-glutamine, 3.7 g/L NaHCO₃, 1% nonessential amino acids, and 10% fetal bovine serum at 37°C in a humidified atmosphere of 95% air and 5% CO₂. Medium was changed every other day. When growing to 80% confluence, the HepG2 cells were seeded in 24-well plastic plates.

Fa2N-4 cell culture of immortalized hepatocytes was performed by the method previously described (11). In brief, Fa2N-4 cells were plated in collagen-coated 24-well plates (0.67 × 10⁶ cells/well, 0.5 mL/well). Cells were maintained at 37°C with 5% CO₂ and 95% relative humidity. After cell attachment (approximately 3h), MFE plating medium was replaced with MFE supporting medium containing supplement A and the cells were cultured for 2 days before treatment.

Assay of CYP3A4 activity in cells

It is well-known that both N-dealkylation of verapamil (6) and 1’-hydroxylation of midazolam (12) were mainly mediated by CYP3A4. The formations of both norverapamil and 1’-hydroxymidazolam were used as the marker to assess CYP3A4 activity. Briefly, the cultured cells were washed once using Hanks’ balanced salt solution (HBSS, pH 7.4) and incubated with 100 μM verapamil or 50 μM midazolam at 37°C for 120 min. The incubation media were collected for assaying norverapamil and 1’-hydroxymidazolam. The amounts of norverapamil and 1’-hydroxymidazolam were measured by HPLC (6) or LC-MS (12).

The cells were further lysed by 3 freeze-thaw cycles and protein concentrations were measured by the Bradford method using BSA as the standard (13). Enzyme activities were expressed as pmol metabolite·min⁻¹·mg protein⁻¹.

Effect of diabetic rat serum on CYP3A4 activity in HepG2 cells

Diabetic rats were induced by a single intraperitoneal injection of 65 mg/kg of STZ dissolved in 0.1 M sodium citrate buffer (pH 4.5) after acclimation for 1 week according to our previously described method. Age-matched control rats only received vehicle. On day 7 after STZ injection, fasting blood glucose levels were measured using a commercially available glucose kit (Jiancheng Biotech Co., Nanjing, China) based on the glucose oxidase method. Rats with fasting blood glucose levels in excess of 11.1 mM were considered to be diabetic. The serum of diabetic rats was collected after 5 weeks following STZ injection. The characteristics of the animals could be found in our previous report (6). The serum was inactivated by heating at 56°C for 30 min and filtered with a 0.22-μm filter before treatment. The concentrations of fatty acids (PA, OA, SA, LA, and AA) in serum were assayed as their 2-nitrophenylhydrazine derivatives by reverse-phase HPLC previously described...
Fatty Acids Induce CYP3A4 in HepG2 Cells

Effects of the abnormal components in diabetic serum on CYP3A4 activity in HepG2 cells

Some components in serum are abnormal under the diabetic condition, including decreased level of insulin and increased levels of glucose, cholesterol, and fatty acids. The effects of abnormally altered components on the CYP3A4 activity in cells were investigated. Briefly, sub-confluent (approximately 80%) HepG2 cells seeded in 24-well plates were incubated in presence of 100% serum from diabetic or control rats for 48 h. The CYP3A4 activity was measured as described above. The cell viability test by MTT assay showed that 48-h exposure to 100% diabetic rat serum or 100% control rat serum used in this study did not damage the viability of cultured cells.

Effects of pharmacological inhibitors on fatty acid-induced CYP3A4 activity in HepG2 cells

Chelerythrine (PKC inhibitor, 0.4, 2, and 10 μM), wortmannin (PI3K/Akt inhibitor; 0.4, 2, and 10 μM), Compound C (AMPK inhibitor; 2, 10, and 50 μM), and PTCD (NF-κB inhibitor; 0.4, 2, and 10 μM) were used to investigate whether these inhibitors reverse the alteration in CYP3A4 activity induced by fatty acids. HepG2 cells were incubated with 0.2 mM PA or OA, in the presence or absence of each pharmacological inhibitor for 48 h.

Western blotting

The protein expression of CYP3A4 in HepG2 cells were determined by western blotting. Homogenate proteins (100 μg) from treated HepG2 cells were separated by sodium dodecyl sulfate–polyacrylamide (10%) gel electrophoresis and transferred onto a PVDF transfer membrane. Nonspecific binding sites were blocked with 5% (w/v) nonfat dried milk in Tween (0.1%) / phosphate-buffered saline (PBS-T, pH 7.4) for 2 h, and then the blots were incubated with human monoclonal antibody to CYP3A4 (1:2500; Abcam, Ltd., Hong Kong) overnight at 4°C. GAPDH (human monoclonal antibody, 1:10,000; Anbo Biotechnology Co., Ltd., San Francisco, CA, USA) was used as an internal control. After washing, membranes were incubated with peroxidase-conjugated anti-rabbit IgG (1:2500; Bioworld Technology Co., Ltd., St. Louis Park, MN, USA) or anti-mouse IgG (1:10000; Cell Signaling Technology Co., Ltd., MN, USA) for 2 h and then washed 4 times with PBS-T. Immunoreactive proteins were visualized by chemiluminescence (ECL Western Blotting Detection Reagents: KGP1123; Nanjing KeyGEN Biotechnology Co., Ltd., Nanjing, China), and band density was measured by densitometry.

QT-PCR analysis

For quantitative comparison of CYP3A4 and PXR mRNA levels, QT-PCR was performed using SYBR-green fluorescence. HepG2 cells (4.0 × 10^5 cells/well) maintained in DMEM supplemented with 10% fetal bovine serum (FBS) were seeded in 6-well plates and incubated at 37°C overnight. Then the cells were incubated with medium containing PA, OA, and positive inducer (rifampicin and VD3) for 48 h. The control cells were treated with vehicle. Total RNA was isolated from the cultured cells using the TRizol reagent (SunShineBio, Nanjing, China) according to the manufacturer’s instructions. Primers used for the amplification of the cDNAs of interest were as follows: for CYP3A4, forward 5′-CAGTTTTTACCAAAATAGGACCAC-3′ and reverse 5′-ATAATCTGAGCGTTTCATTCACCAC-
3′; for PXR, forward 5′-TCAGATCCCACTAAAGTGTCAG-3′ and reverse 5′-GACTCAGGAAGCGAACAAAC-3′; for β-actin, forward 5′-CAGTCGGTTTGGAGCGAGCAT-3′ and reverse 5′-GGACTTCCTGTAACAACGCTCT-3′. The cDNA synthesis reaction was performed from 2 μg total RNA with the PrimeScript™ RT Reagent Kit. mRNA expression was normalized against that of β-actin. TaqMan real time PCR assays were performed on a PTC-200 Thermo Cycler (MJ Research, Waltham, USA). Amplification was performed in 25 μL reaction mixture: 12.5 μL of qPCR Master Mix (Promega, Madison, WI, USA), 2.0 μL of 10 × cDNA, 2.5 μL of 2 μM forward and reverse primers, and 9.5 μL of Nuclease-free water. Each assay was performed in triplicate with β-actin mRNA as the endogenous control. The QT-PCR thermal cycling parameters were as follows: initial denaturation for 2 min at 95°C, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. Melting curves were performed to investigate the specificity of the PCR reaction. Relative quantification (RQ) of each gene expression was calculated according to comparative Ct method using the formula: RQ = 2^{ΔΔCt}.

**Statistical analysis**

All data were expressed as the means ± standard deviation (S.D.). Analysis of variance (ANOVA) was used to determine if the differences between the experimental groups were statistically significant. The 0.05 level of probability was used as the criteria for significance.

**Results**

**Activity of CYP3A4 and its induction in HepG2 cells**

To assess the utility of the HepG2 cells and identify the function of CYP3A4, the activity of CYP3A4 was measured using the formation of norverapamil following 48-h incubation in presence of the inducers (VD3 and rifampicin) or the inhibitors (ketoconazole and erythromycin). Preliminary experiments showed that the formation of norverapamil in HepG2 cells was dependent on both the concentration of verapamil and time of incubation. Therefore, the concentration of verapamil and incubation time were set to 100 μM and 120 min, respectively, for evaluating the CYP3A4 activity. As expected, both CYP3A4 inducers VD3 (0.25 and 0.5 μM) and rifampicin (10 and 20 μM) concentration-dependently increased the formation of norverapamil in HepG2 cells (Fig. 1A). Treatments with 0.25 and 0.5 μM of VD3 led to significant increase in the CYP3A4 activity in HepG2 cells by 1.6- and 2.2-fold of control cells. A significant concentration-dependent increase in CYP3A4 activity was also observed in HepG2 cells treated with rifampicin. It was also found that both CYP3A4 inhibitors ketoconazole (5 and 20 μM) and erythromycin (5 and 20 μM) significantly reduced the CYP3A4 activity in HepG2 cells (Fig. 1B). All the results clearly demonstrated that the HepG2 cells used in the study were suitable for assessing the activity of CYP3A4.
Effect of serum from diabetic rats on CYP3A4 activity in HepG2 cells

The CYP3A4 activity in HepG2 cells treated with serum from diabetic rats was assessed using formation of norverapamil from verapamil (Fig. 1C). The results showed that the 48-h incubation with serum from diabetic rats significantly increased CYP3A4 activity in HepG2 cells, causing it to increase by 83% of that incubated with serum from control rats, inferring existence of some abnormal components inducing CYP3A4 activity in the serum of diabetic rats.

Effects of the abnormal components in diabetic serum on CYP3A4 activity in HepG2 cells

It is generally accepted that diabetes is associated with a decreased level of insulin and increased levels of glucose and cholesterol. In addition, the increased concentrations of free fatty acids in serum often occur. In this study, the concentrations of OA, LA, PA, SA, and AA in the serum of diabetic rats were also measured. It was consistent with our expectation that diabetes significantly increased the concentrations of fatty acids in serum (OA: 1.347 ± 0.397 mM in diabetic rats vs. 0.298 ± 0.088 mM in control rats, \( P < 0.01 \); LA: 0.764 ± 0.300 mM in diabetic rats vs. 0.216 ± 0.064 mM in control rats, \( P < 0.01 \); PA: 0.871 ± 0.384 mM in diabetic rats vs. 0.370 ± 0.083 mM in control rats, \( P < 0.05 \); SA: 1.103 ± 0.950 mM in diabetic rats vs. 0.315 ± 0.033 mM in control rats, \( P < 0.01 \); and AA: 0.185 ± 0.023 mM in diabetic rats vs. 0.152 ± 0.016 mM in control rats, \( P < 0.05 \)).

To identify whether the induction of CYP3A4 activity results from the abnormal alteration in levels of insulin, glucose, cholesterol, or fatty acids, effects of these components on the CYP3A4 activity in HepG2 cells were screened. Contrary to our expectation, both insulin and cholesterol showed little effect on CYP3A4 activity in HepG2 cells. However, PA, OA, SA, and LA increased the CYP3A4 activity in HepG2 cells in a concentration-dependent manner. Forty-eight hour incubation with 0.2 mM of PA, OA, SA, or LA for 48 h, respectively, and then the CYP3A4 activity was detected by the formation of norverapamil; an inducer of CYP3A4, rifampicin (10 \( \mu \)M), was used as a positive control (D). Data are reported as the means ± S.D. from 4 independent experiments. *\( P < 0.05 \), **\( P < 0.01 \).
(Fig. 2B). Induction of CYP3A4 activity in HepG2 cells by both PA and OA was further verified using the formation of 1′-hydroxymidazolam from midazolam (Fig. 2C). Effects of AA on CYP3A4 activity were also investigated, and we found that incubation with 0.2 mM AA did not induce CYP3A4 activity (data not shown). All these results indicated that the increased levels of certain fatty acids in serum of diabetic rats may be one of the main factors inducing activity of CYP3A4 under diabetic state.

The effects of 4 fatty acids on CYP3A4 activity were further verified using Fa2N-4 cells (Fig. 2D). Similar to the findings in HepG2 cells, 48-h incubation with 0.2 mM of PA, OA, SA, and LA enhanced CYP3A4 activity in Fa2N-4 cells.

Effects of PA and OA on protein and mRNA expression of CYP3A4 in HepG2 cells

Accumulated evidences showed that unsaturated fatty acid and saturated fatty acids may have different, sometimes opposite, biochemical or physiological activity or effect. Therefore, we chose OA and PA as representatives of unsaturated fatty acid and saturated fatty acid, respectively, to further study their effects on the induction of CYP3A4 activity. Effects of PA and OA on protein and mRNA expression of CYP3A4 in HepG2 cells were measured by western blotting and QT-PCR, respectively (Figs. 3 and 4). As expected, OA and PA both increased protein and mRNA expressions of the CYP3A4 in HepG2 cells. PA induction on protein and mRNA expressions of CYP3A4 was stronger than that of OA. VD3 and rifampicin, positive inducers of CYP3A4, also strongly induced protein and mRNA expression of CYP3A4. Nuclear receptor pregnane X receptor (PXR) is considered to be a key regulator of CYP3A4 gene transactivations triggered by chemicals. The present results showed that both OA and PA also markedly enhanced mRNA expression of PXR in HepG2 cells.

Effects of pharmacological inhibitors on fatty acid–induced CYP3A4 activity in HepG2 cells

To determine which cell signaling pathways were involved in the fatty acids–mediated induction of CYP3A4 activity, HepG2 cells were incubated with 0.2 mM PA or OA co-administrated with different pharma-
The CYP3A4 activity was evaluated by the metabolism of midazolam. It was interesting to find that the Nf-κB inhibitor PTDC enhanced PA-mediated induction of CYP3A4 activity in a concentration-dependent manner, while attenuating the OA-mediated induction of CYP3A4 activity. Compound C concentration-dependently inhibited both OA and PA inductions of CYP3A4 activity. The activities of CYP3A4 in cells co-treated with 10 μM compound C were only 34% and 71% of cells treated only with PA and OA, respectively. Chelerythrine dose-dependently inhibited the OA-mediated induction of CYP3A4 activity. However, only low concentration of chelerythrine (0.2 μM) could suppress PA-mediated induction of CYP3A4 activity. Wortmannin hardly affected PA-mediated induction of CYP3A4 activity. Although Wortmannin showed a trend to affect OA-mediated induction of CYP3A4 activity, no concentration-dependent manner was observed.

Effects of the pharmacological inhibitors themselves on CYP3A4 activity were also investigated. The results demonstrated that most pharmacological inhibitors with the concentration used could not alter the CYP3A4 activity, but the high concentrations of Compound C (10 μM), chelerythrine (5 μM), and wortmannin (10 μM) significantly influenced the CYP3A4 activity. Significant increases of CYP3A4 activity were observed in 5 μM chelerythrine–treated cells and 10 μM wortmannin–treated cells. However, 10 μM Compound C caused a decrease in the CYP3A4 activity of HepG2 cells.
Discussion

Our previous studies showed that activity and expression of the hepatic CYP3A in diabetic rats were significantly up-regulated, leading to the smaller AUC and higher clearance of verapamil, the substrate of CYP3A4 (6, 7). The present study was designed to identify factors leading to induction of hepatic CYP3A4 activity under the diabetic condition using HepG2 cell lines as an in vitro model. The activity of hepatic CYP3A4 was evaluated using formation of norverapamil from verapamil and 1'-OH midazolam from midazolam. The main finding was that serum from diabetic rats significantly increased hepatic CYP3A4 activity. Further studies showed that the induction was attributed to the increased concentrations of free fatty acids in serum.

Primary human hepatocytes are considered to be the gold standard model for xenobiotic metabolism, but large interindividual donor variability, rapid loss of drug-metabolizing enzyme expression, poor availability, and high cost have limited its application (17, 18). Several human hepatic cell lines (e.g., HepG2, LS180, Fa2N-4, and HepaRG) have been introduced to overcome the previously mentioned drawbacks despite the lower constitutive levels of drug-metabolizing enzymes in these cell lines relative to human hepatocytes. The HepG2 cell line is one of the most widely used human hepatoma cells. HepG2 cells possess both phase I and II enzymes, and it has been verified as an in vitro model to assess the induction of CYP3A4 activity by several studies (19 – 22). But the activity of this enzyme is dependent on the source and culture conditions (23). Therefore, HepG2 cells routinely used in assays in vitro should be firstly characterized for their drug-metabolizing capabilities. At the beginning of this study, the activity of hepatic CYP3A4 was confirmed using two probe substrates of CYP3A4, verapamil and midazolam. The activity of hepatic CYP3A4 could be both induced by CYP3A4 inducers (rifampicin and VD3) and inhibited by CYP3A4 inhibitors (ketoconazole and erythromycin). All these results clearly demonstrated that the HepG2 cell line used in the study was suitable for assessing of hepatic CYP3A4 activity.

Diabetic serum–treated cells were widely used to investigate the impact of diabetes on cells in vitro, including rabbit aortic myomedial cells (24), rat embryos (25), and human endothelial cells (26). We also used the co-cultured rat brain microvessel endothelial cell monolayers with 100% diabetic rat serum as in vitro diabetic model to examine the effect of the diabetes on the function of P-glycoprotein (15) and breast-cancer resistance protein (27) in the blood–brain barrier. Here, HepG2 cells were co-cultured with 100% diabetic serum as in vitro diabetic model to investigate effects of serum from diabetic rats on hepatic CYP3A4 activity. As expected, the incubation with serum from diabetic rats significantly increased CYP3A4 activity, inferring the existence of some components inducing CYP3A4 activity.

It is generally accepted that diabetes, apart from the decreased level of insulin and increased level of glucose in serum, is also associated with increased levels of cholesterol and fatty acids. In order to investigate whether alterations in these components contribute to induction of CYP3A4 activity, the effects of these components on CYP3A4 activity in HepG2 cells were studied separately. It was found that cholesterol had little influence on the activity of CYP3A4. In addition, insulin treatment was reported to reverse the alteration in hepatic CYP450s induced by diabetes (28), and absence of insulin led to a dramatic decrease in the levels of CYP2E1 and CYP2B mRNA and protein level in primarily cultured rat hepatocytes. However, data from the present study showed that insulin itself did not affect hepatic CYP3A4 activity, which was in agreement with Woodcroft’s report in primarily cultured rat hepatocytes (29). Similarly, Woodcroft et al. reported that absence of insulin attenuated induction of phenobarbital-induced CYP3A in hepatocytes (30). In terms of the contribution of glucose, we discovered that physiological or pathological levels of glucose (5.5, 25 mM) failed to affect the activity of CYP3A4 in HepG2 cells, while a much higher glucose level (68 mM) reduced CYP3A4 activity in HepG2 cells without affecting the cell viability (31), inferring that induction of CYP3A4 did not result from the increased level of glucose. Several studies have showed that consumption of glucose or high carbohydrate diet decreased metabolism of some drugs such as barbiturates, antipyrine, and theophylline (32, 33). In vitro, co-culture with high glucose (25 mM) also reduced CYP450 enzyme activity in endothelial cells via elevating level of superoxide (34). All these results indicated that alterations in levels of insulin, cholesterol, and glucose seemed not to be main factors inducing hepatic CYP3A4 activity in serum from diabetic rats.

Altered level of serum free fatty acids occurs in numerous diseases related to abnormal lipid metabolisms, including diabetes, obesity, and nonalcoholic fatty liver disease. Our current research discovered that the concentrations of OA, LA, PA, and SA in serum of diabetic rats were several times higher than those in normal rats. Several studies have showed that the unsaturated fatty acids, not saturated fatty acids, inhibited CYP3A4-catalyzed metabolic reactions (35, 36). It was reported that 14-h treatment with a high-concentration fatty acids mixture (1 and 2 mM) significantly decreased...
CYP3A4 activity in primarily cultured human hepatocytes (37). However, data from the present study showed that 48-h treatment of hepatic cells (both HepG2 and Fa2N-4 cells) with PA and OA (0.1 and 0.2 mM) induced CYP3A4 activity. The discrepancy may result from different incubation time and concentration of fatty acids being used. High concentration of fatty acids was reported to induce the accumulation of lipids and apoptosis in hepatocytes (38). Our preliminary study also showed that fatty acid at higher than 0.2 mM could significantly decrease the cell viability after 48-h treatment (data not shown). It was reported that the impact of diabetes on CYP450 also has a relationship with the duration of the disease, and long-term diabetes resulted in more significant changes of CYP450 (39, 40). These findings indicated that treatment of cells with a high level of fatty acids for long time may be better for investigating the effect of fatty acids in an in vitro study. It was also found that that the expressions of CYP3A4 and PXR mRNA in HepG2 cells could be induced by both PA and OA, and induction of CYP3A4 by chemical agents is usually via activating PXR, inferring that induction of CYP3A4 by fatty acids may be involved in PXR. All the results suggested that induction of CYP3A4 activity was, at least in part, due to the increased levels of fatty acids in the serum of diabetic rats. Our previous studies showed the expression and activity of CYP3A2 were increased in the liver of type 2 diabetic rats and rats fed with high-fat diet (7). Irizar et al. also reported that CYP3A level was higher in the obese Zucker rat when compared with the lean littermate (41). In the liver of rats with non-alcoholic steatohepatitis, CYP3A11 was significantly enhanced (42), but the CYP3A was reduced in rats with non-alcoholic fatty liver disease (43). The above studies demonstrated that lipid metabolism disorders could alter the activity and expression of CYP3A4. Besides fatty acid, there are many other abnormal factors in diabetic serum such as ketone body and hormones (growth hormone and testosterone levels). It has been reported that ketone body increased the expression of CYP2B in primary cultured rat hepatocytes (44), and the elevation of ketone body plays a critical role in the up-regulation of hepatic CYP2E1 in diabetic rats (45). Impaired secretion of growth hormone in diabetic rats was also responsible for the regulation of CYP450. Testosterone replacement treatments could significantly reverse the changes in some CYP450 isoforms in the diabetic male rats (46). Hepatic CYP2E1 increased in male rat treated by hypophysectomy, and administration of growth hormone to these rats reversed the elevated hepatic CYP2E1 (9). These factors may also be involved in induction of CYP3A4 activity and need further study.

To study mechanisms by which free fatty acids cause CYP3A induction, we investigate the role of several signalling pathways on the CYP3A regulation, such as PI3K, AMPK, PKC, and NF-κB, which have been reported to participate in the fatty acid-mediated regulation (47 – 51). The PI3K inhibitor, wortmannin, ameliorated the insulin-mediated decrease in CYP2E1 mRNA level (30, 52), inferring PI3K as an obligatory component in the suppression of CYP2E1 expression by insulin. Similarly, both the PI3K and PKC inhibitor also ameliorated the suppressive effect of acetoacetate on CYP2E1 mRNA expression (53). There was a report showing that NF-κB plays a pivotal role in the regulation of CYP3A4 through interactions of NF-κB with the PXR-retinoid X receptor (RXR) complex (54) or the impact on CYP3A4 protein stability (55). Moreover, fatty acid–induced muscle insulin resistance and glucose uptake dysfunction were involved in PKC activation and oxidative stress–activated signaling pathways (56). All the results suggested that these signal pathways may be involved in the induction of CYP3A4 by fatty acids in HepG2 cells. To verify the deduction, the effects of these pharmacological inhibitors on induction of CYP3A4 in HepG2 cells by OA and PA were documented. The results showed that effects of these pharmacological inhibitors on induction of CYP3A4 by OA were different from those by PA. The inhibitors of PKC (chelerychrine) ameliorated the induction of CYP3A4 by PA and OA, showing stronger inhibition on induction of CYP3A4 activity by PA. The inhibitor of NF-κB (PTDC) enhanced induction of CYP3A4 activity by PA, while it inhibited that by OA. The PI3K inhibitor (wortmannin) hardly affected induction of CYP3A4 by PA. Several studies demonstrated that PA and OA induced both NF-κB (57, 58) and PKC activities (59, 60), and the stimulatory effects of OA on the PKC pathway was stronger than those of PA (61, 62). PA was also reported to inhibit phosphorylation of AMPK (63, 64) and Akt (65, 66). In sharp contrast, OA enhanced the activity of Akt (67) and showed weak inhibition on phosphorylation of AMPK (68). All these may explain why that effects of these pharmacological inhibitors on the induction of CYP3A4 by OA were different from those by PA. In light of the above, our present study provided direct evidence that induction of CYP3A4 activity by OA may be partly via AMPK-, PKC-, and NF-κB–dependent pathways, and the induction by PA was possibly associated with a PKC-dependent pathway. The real relationship between the signal pathways and the induction of CYP3A4 activity by PA and OA is still obscure and requires further investigation.

In conclusion, the present study clearly demonstrated that the increased levels of fatty acids in serum from
diabetic rats contributed to induction of CYP3A4 expression and activity in hepatic cells, which was one of the factors leading to the up-regulation of CYP3A4 in diabetic conditions. In addition, the signaling pathways participating in the regulation of CYP3A4 by PA (saturated fatty acid) may be different from that by OA (unsaturated fatty acid).

Acknowledgment

This work was supported by funding for National Science Foundation of China (No. 81273587, 81373482, 81072693), National Youth Science Foundation of China (No. 81102503), National Science Foundation of China (No. 81273587, 81373482, 81072693), National Basic Research Program of China (973 Program) (2011CB505300, 2011CB505303) and the Fundamental Research Funds for the Central Universities.

References

Fatty Acids Induce CYP3A4 in HepG2 Cells


