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Involvement of pregnane X receptor in the suppression of carboxylesterases by metformin in vivo and in vitro, mediated by the activation of AMPK and JNK signaling pathway

Enfang Shan, Zhu Zhu, Shuangcheng He, Dongbao Chu, Dinghao Ge, Yunran Zhan, Wei Liu, Jian Yang *, Jing Xiong *

Department of Pharmacology, Nanjing Medical University, Nanjing, Jiangsu 210029, China

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Irinotecan hydrochloride (PubChem CID: 74990)
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ABSTRACT

Type 2 diabetes mellitus (T2D) is a complex metabolic disorder requiring polypharmacy treatment in clinic, with metformin being widely used antihyperglycemic drug. However, the mechanisms of metformin as a perpetrator inducing potential drug-drug interactions and adverse drug reactions are scarcely known to date. Carboxylesterases (CESs) are major hydrolytic enzymes highly expressed in the liver, including mouse carboxylesterase 1d (Ces1d) and Ces1e. In the present study, experiments are designed to investigate the effects and mechanisms of metformin on Ces1d and Ces1e in vivo and in vitro. In results, metformin suppresses the expression and activity of Ces1d and Ces1e in a dose- and time-dependent manner. The decreased expression of nuclear receptor PXR and its target gene P-gp indicates the involvements of PXR in the suppressed expression of carboxylesterases by metformin. Furthermore, metformin significantly suppresses the phosphorylation of AMPK and JNK, and the suppression of carboxylesterases induced by metformin is repeatedly abolished by AMPK inhibitor Compound C and JNK inhibitor SP600125. It implies that the activation of AMPK and JNK pathways mediates the suppression of carboxylesterases by metformin. The findings deserve further elucidation including clinical trials and have a potential to make contribution for the rational medication in the treatment of T2D patients.

1. Introduction

Type 2 diabetes mellitus (T2D) is a metabolic disorder, which is characterized by chronic hyperglycemia and insulin resistance, affecting a large amount of the global population with potentially serious health outcomes (Ginter & Simko, 2012). Since polypharmacy treatment is always required for the T2D patients, biotransformation characteristics and potential drug-drug interactions in patients deserve full elucidation (Breuker et al., 2017). Metformin, a historical antihyperglycemic drug, contributes to lifespan extension, treatment and prevention of sedentariness damages, insulin resistance, and obesity for T2D patients (Senesi et al., 2016). Considering the extended clinical use of metformin (Loos et al., 2017; Li et al., 2016; Castillo-Quan & Blackwell, 2016), the risks of unwanted drug-drug interactions and drug adverse reactions associated with metformin-based polypharmacy treatment for patients should be thoroughly evaluated. However, previous studies predominantly focus on how other drugs may affect the pharmacokinetics of metformin as a victim (Zack et al., 2015; El Messaoudi et al., 2016), with limited data available regarding the influence of metformin as a perpetrator on the metabolism and clearance of other co-administered drugs.

In all factors that may alter the hepatic capacity of drug metabolism, regulated expression of Drug-metabolizing enzymes (DMEs) and drug transporters contributes the most. Carboxylesterases (CESs) represent an important group of enzymes that have highly abundant expression in the liver. The drugs being hydrolyzed by CESs contain such functional

* Correspondence authors.
E-mail addresses: jianyang@njmu.edu.cn (J. Yang), xiong.jing@njmu.edu.cn (J. Xiong).

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groups as carboxylic acid ester, amide and thioester, and occupy about 20% of therapeutic agents in clinic (Xiao et al., 2012). There are two major CESs expressed in the liver, human carboxylesterase 1 (CES1) and carboxylesterase 2 (CES2) (Xiong et al., 2014a). Pregnane X receptor (PXR) is one of the most important nuclear receptors regulating the transcriptional expression of DMEs and drug transporters, including Cytochrome P450 3A4 (CYP3A4), multidrug resistance 1 (MDR1)-encoded P-glycoprotein (P-gp), as well as CESs (Luo et al., 2017; Pondugula et al., 2015). Upon activation, PXR is translocated from the cytoplasm to the nuclear compartment, heterodimerizes with the retinoid X receptor (RXR) and upregulates the transcription by binding to the response element in the promoter of target DMEs and drug transporters (Gu et al., 2006).

A number of pathways including AMP-activated protein kinase (AMPK) and c-Jun N-terminal kinase (JNK) are involved in the regulation of PXR and its target genes (Kumari et al., 2015; Krausova et al., 2011). As a known activator of AMPK, metformin appears to exert its pharmacological actions both AMPK-dependently and -independently (Lee et al., 2012; Do et al., 2013). It is also reported that AMPK interacts with transcription factors apart from PXR (Sozio et al., 2011) and disturbs the expression of PXR-target genes (Wang et al., 2015). In addition, involvement of the JNK pathway in the actions of metformin has also been recently reported, as the activation of JNK pathway is involved in metformin-induced apoptosis in lung cancer cells (Wu et al., 2011).

As mouse carboxylesterase 1d (Ces1d) and Ces1e strongly cross-react with human CES1 and CES2 respectively (Xiao et al., 2012), experiments are designed to investigate the effect of metformin on the expression and activity of mouse hepatic Ces1d and Ces1e both in vivo and in vitro. Based on the above information, nuclear receptor PXR, AMPK and JNK signaling pathways are hypothesized to mediate the regulation of CESs by metformin. The findings could make a great contribution to move a step in guiding clinical rational drug use for the T2D patients and deserve further investigations including clinical trials.

2. Materials and methods

2.1. Materials

Metformin hydrochloride (MET) was purchased from Aladdin (Shanghai, China). Para-nitrophenylacetate (PNPA), clopidogrel bisulfate, irinotecan hydrochloride, AMPK inhibitor 6-[4-(2-piperidin-1-ylethoxy) phenyl]-3-pyridin-4-ylpyrazolo [1,5-a] pyrimidine (Compound C), JNK inhibitor SP600125 and Rhodamine 123 (Rho123) were from Sigma (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) was from Invitrogen (Carlsbad, CA, USA); and fetal bovine serum (FBS) was from Hyclone Laboratories (Logan, UT, USA). Mouse Ces1d or Ces1e was detected by antibody against human CES1 or human CES2, kindly provided by Dr. Bingfang Yan (Xiong et al., 2014b). Antibody against mouse PXR was also donated by Dr. Yan. Antibodies against pAMPK, AMPK, pJNK, JNK and β-actin were from Bioworld (St. Louis Park, USA), antibody against P-gp was from Abcam (Cambridge, UK). The goat anti-rabbit or anti-mouse IgG conjugated with horseradish peroxidase was from Pierce Chemical (Pierce, Rockford, IL, USA). All other reagents were of analytical grade and commercially available. All the experiments for this study were conducted in compliance with the principles of Good Laboratory Practice (GLP) standards for Nonclinical Laboratory Studies.

2.2. Animal experiments

The use of animals was approved by IACUC (Institutional Animal Care and Use Committee) of Nanjing Medical University. To demonstrate the in vivo effects of metformin on the expression of mouse Ces1d and Ces1e in the liver, male Institute of Cancer Research (ICR) mice (20–25 g) obtained from Yangzhou University (Yangzhou, China) were housed individually in animal cages in a room with controlled temperature (22 ± 1 °C), humidity (50 ± 10%), and lighting (lights on from 8:00 to 20:00). After 1 week of acclimatization, metformin was dissolved in distilled water and administered to mice by oral gavage (0, 100 or 300 mg/kg/day for 3 days, or 300 mg/kg/day for 0, 1 or 3 days). The dosage for mice is converted from human dosage using body surface area normalization (Reagan-Shaw et al., 2008), and refers to previous studies (Krausova et al., 2011; Pham et al., 2017; Xu et al., 2015). 24 h after the last administration of drug or vehicle, mice were anesthetized by injection with urethane (1 g/kg body weight). The liver was perfused with PBS through the portal vein to remove blood and frozen at −80 °C for preparing S9 fractions. Every effort was made to minimize animal suffering and to reduce the number of animals used for the experiments.

2.3. Preparation of S9 fractions

The frozen livers were thawed in homogenization buffer (50 mM Tris–HCl, pH 7.4, 150 mM KCl and 2 mM EDTA) and then homogenized with six passes of Teflon pestle driven by a Wharton stirrer. The homogenates were centrifuged at 10,000 × *g* for 20 min at 4 °C. The S9 fractions of liver (supernatant) were assayed for the hydrolysis of PNPA and for protein expression.

2.4. Primary mouse hepatocyte culture and treatment

Mouse hepatocytes were isolated from livers of male ICR mice here referred to as modification of the two-step perfusion method, described previously (Xiong et al., 2014a). Isolated hepatocytes were suspended in the DMEM supplemented with 10% FBS, seeded into collagen-coated six-well plates and maintained at 37 °C for 4 h to allow attachment. After being continuously cultured for one day with a change of fresh medium, primary mouse hepatocytes were treated with MET (0, 0.125, 0.25 or 0.5 mM) for 24 h.

2.5. Enzyme activity assay

The overall hydrolytic activity was tested using standard substrate PNPA by spectrophotometer. After the treatment with metformin (0, 0.125, 0.25, and 0.5 mM), cells were rinsed with D-Hank's and harvested in 80 μL of 100 mM potassium phosphate buffer (pH 7.4). The cell suspension was sonicated by a sonifier (Nanjing, China). Then the supernatants were obtained by precipitation using centrifugation at 12,000 × *g* for 15 min at 4 °C, and assayed for overall hydrolytic activity toward PNPA as described previously (Xiong et al., 2014a). A sample tube (1 mL) contained 10 μg of cell lysates in 100 mM potassium phosphate buffer (pH 7.4), 10 μL PNPA and substrate (1 mM) at 37 °C. The addition of PNPA was used to initiate the reactions and the hydrolytic rate was recorded from an increase in absorbance at 400 nm. The extinction coefficient (E400) was determined to be 13 mM^{-1} cm^{-1}. Several controls were conducted including incubation without proteins.

2.6. Cytotoxicity and morphologic assay

Primary mouse hepatocytes were seeded into 96-well plates at the density of 5000 cells each well. After continued culture for one day, the medium was replaced with fresh medium with or without 0.5 mM metformin for 12 h. After that, the cells were washed with DMEM twice and treated with different concentrations of clopidogrel (0, 10, 30 or 100 μM) for 30 h or irinotecan (0, 10, 30 or 100 μM) for 24 h. The medium was replaced with fresh medium containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) at a final concentration of 0.5 mg/mL. After 4 h incubation at 37 °C, the medium was gently decanted, and dimethyl sulfoxide was added to dissolve formazan product. The optical density (OD) was determined at 570 nm, and the final OD values were expressed by subtracting the