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Glucose dominates the regulation of carboxylesterases induced by lipopolysaccharide or interleukin-6 in primary mouse hepatocytes

Jing Xiong, Wei Shang, Lili Wu, Ruini Chen, Wei Liu, Rui Ning, Gang Hu, Jian Yang *

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

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ABSTRACT

Aims: Altered drug disposition has been associated with inflammation and diabetes, leading to the alteration of drug efficacy and toxicity. Carboxylesterases are major hydrolytic enzymes in the liver, catalyzing the hydrolytic biotransformation of numerous therapeutic agents. Therefore, how glucose affects the regulation of carboxylesterases by interleukin-6 (IL-6) and lipopolysaccharide (LPS) were investigated.

Main methods: Primary mouse hepatocytes were cultured. Protein levels were measured by Western blot or enzyme linked immunosorbent assay (ELISA), while confocal laser scanning microscope and flow cytometry were used to confirm the activation of pregnane X receptor (PXR). Carboxylesterase activity was evaluated by enzymatic and toxicological assays.

Key findings: Elevated glucose (11 or 25 mM) significantly increased carboxylesterase expression compared to 5.6 mM glucose. Carboxylesterase expression and activity were inhibited by LPS or IL-6 in 25 mM glucose, but stimulated in 5.6 mM glucose. The altered expression of carboxylesterases was not consistent with the activation of nuclear factor kappa B (NFκB) but repeatedly with the expression and activation of pregnane X receptor (PXR). The altered activation of PXR was further evidenced by the differential subcellular translocation and the expression of its target gene multidrug resistance 1 (MDR1). It implies that PXR, instead of inflammatory signaling, mediates the regulation of carboxylesterases by inflammatory mediators in different glucose concentrations.

Significance: The findings contribute to clarify the regulation of carboxylesterases by inflammatory mediators, and indicate that carboxylesterase-involved drug metabolism and drug–drug interactions in diabetes should be reevaluated according to the intensity of inflammatory reactions and hyperglycemia.

Introduction

Diabetes mellitus is a complex metabolic disorder that affects a significant fraction of the global population with potentially serious health outcomes (Singh et al., 2004). More drugs are prescribed to diabetic patients than age-matched nondiabetic people, therefore, biotransformation characteristics in patients with diabetes deserve better understanding (Isacson and Stalhammar, 1987; Wandell and Gafvels, 2002). Expression and activity of hepatic drug-metabolizing enzymes (DMEs) can be profoundly altered in diabetes, but the mechanisms are scarcely known. It is shown that diabetes is associated with a significant decrease in hepatic CYP 3A4 without knowing the type of diabetes and diabetes treatment (Dostalek et al., 2011). In contrast, uncontrolled diabetes in experimental animal models results in enhanced expression of several CYP450 isoforms (CYP1A1, 1A2, 1B1, 2B1, 2C12, 2E1, 3A4 and 3A1) and suppressed expression of CYP 2C11, 2C13, 2A2 and 3A2 (Baek et al., 2006; Kim et al., 2005; Lee et al., 2007; Sindhu et al., 2006). An increase in CYP1A2 activity is also observed in patients with type 1 diabetes mellitus (Matzke et al., 2000). These contradictory observations require better understanding on how the energy status of hepatocytes regulates DMEs.

Carboxylesterases (E.C. 3.1.1.1) are major enzymes that hydrolyze drugs containing such functional groups as esters, amides, thioesters and carbamates (Sanghani et al., 2009; Xiao et al., 2012). About 20% of therapeutic agents undergo hydrolytic biotransformation (Xiao et al., 2012). The liver expresses two major carboxylesterases, including human carboxylesterases 1 (CES1) and human carboxylesterases 2 (CES2), whereas the gastrointestinal tract expresses predominantly CES2 (Satoh and Hosokawa, 2006). Although many factors may alter the hepatic capacity of drug metabolism, regulated expression of DMEs contributes the most to the alteration (Parkinson, 2001; Poso and Honkakoski, 2006). Transactivation by nuclear receptors such as the pregnane X receptor (PXR) and constitutive androstane receptor (CAR) is largely responsible for the increased expression of DMEs and drug transporter genes, including multidrug resistance 1 (MDR1)-encoded P-glycoprotein (P-gp) and carboxylesterases (Poso and

Abbreviations: LPS, lipopolysaccharide; IL-6, interleukin-6; DMEs, drug-metabolizing enzymes; Ces (mouse) or CES (human), carboxylesterases; CYP, cytochrome P450; PXR, pregnane X receptor; CAR, constitutive androstane receptor; RXR, retinoid X receptor; NFκB, nuclear factor kappa B; MDR1, multidrug resistance 1; P-gp, P-glycoprotein, DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme linked immunosorbent assay.

* Corresponding author at: Department of Pharmacology, Nanjing Medical University, Nanjing, Jiangsu 210029, China. Tel./fax: +86 25 86863159.
E-mail address: jianyang@njmu.edu.cn (J. Yang).

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Honkakoski, 2006; Staudinger et al., 2010). Upon activation, the nuclear receptors translocate from the cytoplasm to the nuclear compartment, heterodimerize with the retinoid X receptor (RXR) and positively regulate gene expression by binding to the response element in the promoter of target genes (Gu et al., 2006).

Diabetes is now considered as an obesity-linked inflammatory disease (Wellen and Hotamisligil, 2005), with various inflammatory mediators elevated in the system (Hu et al., 2004). Toll-like receptor activator, lipopolysaccharide (LPS), activates nuclear factor kappa B (NFκB) and thus induces the expression of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6). Unlike TNF-α, IL-6 initiates cellular events including the activation of JAK (Janus Kinase) kinases, phosphorylation of STAT (Signal Transducers and Activators of Transcription) transcription factors, and activation of gene transcription (Heinrich et al., 1998). It has been shown that both LPS and IL-6 are capable to downregulate the expression of a variety of DMEs (Gu et al., 2006; Myers et al., 2010), including human CES1 and CES2 (Mao et al., 2011; Yang et al., 2007). In addition to DMEs, many cytokines decrease the expression of drug transporters as well as nuclear receptors that support the transactivation of genes encoding DMEs or transporters (Blokzijl et al., 2007; Cressman et al., 2012; Malekshah et al., 2012). However, the mechanisms of the regulation by LPS and proinflammatory cytokines largely remain unclear.

The present study reports that carboxylesterase expression and hydrolytic activity which responded to LPS or IL-6 are dependent upon the glucose concentration. After evaluating the activation of inflammatory signaling, we further investigate whether PXR mediates the differential regulation of carboxylesterases in different glucose concentrations. The findings could make a contribution to move a further step in understanding the drug disposition alterations in diabetes.

Materials and methods

Materials

LPS from *Escherichia coli* 055:B5, and Rhodamine 123 (Rho123) were purchased from Sigma (St. Louis, MO, USA); and IL-6 was from R&D Systems (Minneapolis, MN). Low glucose (5.6 mM) Dulbecco's modified Eagle's medium (DMEM), was from Invitrogen (Carlsbad, CA, USA). DMEM with respective glucose concentration (11 or 25 mM) was made by adding extra D-glucose into 5.6 mM DMEM. Mouse Ces1d or Ces1e was detected by antibody against human CES1 or human CES2, kindly provided by Dr. Bingfang Yan, because mouse Ces1d or Ces1e can be detected by human CES1 or CES2 antibodies as reported previously (Xiao et al., 2012). Antibody against mouse PXR was also donated by Dr. Yan. Antibodies against p65 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.

Primary mouse hepatocyte culture and treatment

Male ICR mice, 18–22 g, were obtained from the experimental animal center of Nanjing (Nanjing, China). The use of animals was approved by IACUC (Institutional Animal Care and Use Committee) of Nanjing Medical University. Every effort was made to minimize animal suffering and to reduce the number of animals used for experiments. Hepatocytes were isolated from livers of male ICR mice referred to a modification of the two-step perfusion method as we described previously (Feng et al., 2012). Following, cells were suspended in the DMEM (25 mM glucose) supplemented with 10% FBS and seeded into collagen-coated six-well plates and were maintained at 37 °C, in a humidified atmosphere of 5% CO_2 for 4 h to allow attachment. Cells were then washed with PBS and changed with fetal bovine serum free medium. After continued culture for two days, primary mouse hepatocytes were pretreated with indicated concentrations of D-glucose (5.6, 11, or 25 mM) in DMEM for 24 h. Then, saline, LPS (1 μg/mL) or IL-6 (20 ng/mL) was added into respective wells and incubated for 24 h to detect the expression and activation of interest proteins.

Preparation of cytoplasmic and nuclear extracts

Cytoplasmic and nuclear extracts were prepared as described (Sfikas et al., 2012). Briefly, cells were scraped from dishes in PBS, pelleted, washed in hypotonic buffer (10 mM HEPES buffer, pH 7.9, 1.5 mM $MgCl_2$, 5 mM KCl, 1 mM PMSF, 1 mM dithiothreitol, 1 mM Na_3VO_4, 1 mM NaF), and lysed by resuspension in the same buffer with 0.1% Nonidet P-40. Cytoplasmic extracts were isolated by centrifugation at 10,000 ×*g* for 10 min. Nuclear pellets were washed in hypotonic buffer and resuspended in cold extraction buffer (20 mM HEPES, 25% glycerol, 450 mM KCl, 1 mM EDTA, 1 mM PMSF, 1 mM Na_3VO_4, 1 mM NaF), gently agitated at 4 °C for 45 min, and spun at 13,000 ×*g* for 30 min at 4 °C. Supernatants were collected, and protein concentrations were determined with BCA protein assay based on the albumin standard (Pears, Rockford, IL, USA).

Western blotting

Equal amounts of protein were separated on a 12% SDS-polyacrylamide gel and transferred electrophoretically onto polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked with 5% non-fat milk in Tris-buffered saline/0.1% Tween 20 for 2 h, subsequently blotted with respective primary antibodies overnight, and then blotted with horseradish peroxidase-conjugated secondary antibody for 1 h. The protein bands were visualized with enhanced chemiluminescence detection system. Protein levels were quantified by density analysis using Image J software (NIH), and expressed as fold change of interest protein/β-actin.

Cytotoxicity and morphologic assay

Primary mouse hepatocytes were seeded into 96-well plates at the density of 10,000 cells/well. After continued culture for two days, cells were pretreated with indicated concentrations of D-glucose (5.6 or 25 mM) in DMEM for 24 h. Then, saline, LPS (1 μg/mL) or IL-6 (20 ng/mL) was added into respective wells and incubated for 24 h. The cells were then washed with DMEM of respective glucose concentrations once and treated with irinotecan (30 or 100 μM). When the cells were treated for 32 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 2 h incubation at 37 °C, the medium was gently decanted, and dimethyl sulfoxide (150 μL/well) 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 background reading (no seeded cells). Morphologic changes were detected under microscope before MTT assay.

Enzymatic assay

After treatments with saline, LPS (1 μg/mL) or IL-6 (20 ng/mL), cells were rinsed with PBS and harvested in 50 μL of 100 mM potassium phosphate buffer, pH 7.4. The cell suspension was sonicated by a sonifier (Nanjing, China), and the cell debris was removed by centrifugation at 12,000 ×*g* for 15 min at 4 °C. The supernatants were assayed for hydrolytic activity toward para-nitrophenylacetate as described previously (Yang et al., 2007). A sample cuvette (1 mL) contained 10 μg of cell lysates in 100 mM potassium phosphate buffer pH 7.4, and 1 mM substrate at room temperature. Reactions were initiated by adding para-nitrophenylacetate (10 μL of 100 mM stock in acetonitrile), and the hydrolytic rate was recorded from an increase in absorbance at