BBA - General Subjects 1862 (2018) 2261–2270

Contents lists available at ScienceDirect

BBA - General Subjects

journal homepage: www.elsevier.com/locate/bbagen

Structural basis for the hepatoprotective effects of antihypertensive 1,4-dihydropyridine drugs

Yijuan Wei[1], Yi Lu[1], Yanlin Zhu[1], Weili Zheng, Fusheng Guo, Benqiang Yao, Shuangshuang Xu, Yumeng Wang, Lihua Jin*, Yong Li*

State Key Laboratory of Cellular Stress Biology, School of Life Sciences, Innovation Center for Cell Signaling Network, Xiamen University, Xiamen, Fujian 361005, China

ARTICLE INFO

Keywords:
Drug discovery
Structure-activity relationship
Target
Nuclear receptor
Drug repositioning
Antihypertensive drugs

ABSTRACT

Background: The 1,4-dihydropyridines (DHPs) are one of the most frequently prescribed classes of antihypertensive monotherapeutic agents worldwide. In addition to treating hypertension, DHPs also exert other beneficial effects, including hepatoprotective effects. However, the mechanism underlying the hepatoprotection remains unclear.

Methods: Biochemical AlphaScreen and cell-based reporter assays were employed to detect the activities of DHPs towards FXR. A crystallographic analysis was adopted to study the binding modes of four DHPs in complex with FXR. Acetaminophen (APAP)-treated wild-type and FXR knockout mice were used to investigate the functional dependence of the effects of the selected DHPs on FXR.

Results: A series of DHPs were uncovered as FXR ligands with different activities for FXR, suggesting FXR might serve as an alternative drug target for DHPs. The structural analysis illustrated the specific three-blade propeller binding modes of four DHPs to FXR and explained the detailed mechanisms by which DHPs bind to and are recognized by FXR. The results in mice demonstrated that cilnidipine protected the liver from APAP-induced injury in an FXR-dependent manner.

Conclusions: This study reports the crystal structures of FXR in complex with four DHPs, and confirms that DHPs exert hepatoprotection by targeting FXR.

General significance: Our research not only reveals valuable insight for the design and development of next-generation Ca^{2+} blocker drugs to provide safer and more effective treatments for cardiovascular disorders but also provides a novel and safe structural template for the development of drugs targeting FXR. Moreover, DHPs might be potentially repurposed to treat FXR-mediated diseases other than hypertension.

1. Introduction

The 1,4-dihydropyridines (DHPs) are a class of calcium channel blockers (CCBs) that are widely used to treat cardiovascular disorders, including hypertension and angina [1]. By binding to the external, lipid-facing surface of the voltage-dependent Ca^{2+} channel, dihydropyridines allosterically induce an asymmetric conformation of the channel, leading to the channel pore blocked by partially dehydrated Ca^{2+} [2]. The DHP class of CCBs inhibits the opening of L-type calcium channels on vascular smooth muscle cells and the myocardium, resulting in reduced systemic vascular resistance and arterial pressure [3]. Some DHPs also exert their clinical actions by blocking other subtypes of Ca^{2+} channels, such as N-type Ca^{2+} channels [4]. The DHP class of CCBs has become one of the most frequently prescribed antihypertensive monotherapeutic agents worldwide. In addition to their therapeutic uses in treating hypertension, DHPs also exert other beneficial effects. As CCBs, DHPs have been reported to protect against in vivo liver injury induced by a variety of chemicals, including carbon tetrachloride (CCl_4), chloroform, dimethylnitrosamine, thioacetamide and acetaminophen (APAP) [5–8]. Although various clues have been reported, the mechanism underlying the hepatoprotective effects of DHPs remains unclear.

The nuclear bile acid receptor, farnesoid X receptor (FXR, NR1H4), is a member of the ligand-activated transcription factor superfamily. FXR is expressed at high levels in the mammalian liver, intestine, kidney, and adrenal glands [9–11]. By binding to specific ligands, FXR regulates the transcription of a series of target genes involved in a variety of biological and pathological processes, including bile acid and

* Corresponding authors.
E-mail addresses: jinlh@xmu.edu.cn (L. Jin), yongli@xmu.edu.cn (Y. Li).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.bbagen.2018.07.022
Received 10 May 2018; Received in revised form 18 July 2018; Accepted 20 July 2018
Available online 23 July 2018

cholesterol homeostasis, glucose and lipid metabolism, liver regeneration, hepatic inflammation and injury, and hepatocellular carcinoma [12–16]. FXR plays particularly important roles in protecting the liver from bile acid-induced hepatotoxicity and chemical-induced liver injury [17]. Regarding endogenous bile acid toxicity, FXR controls and fine-tunes bile acid homeostasis and induces bile acid efflux and the activation of the detoxification machinery by regulating genes involved in bile acid synthesis, transport and uptake [18]. Regarding exogenous chemical-induced injury, FXR has been reported to induce the expression of genes involved in phase II (conjugation) and phase III (elimination) xenobiotic metabolism in mice [19, 20]. Following target gene regulation, agonist-activated FXR increases hepatic glutathione (GSH) levels, reduces the levels of lactate dehydrogenase (LDH), alanine aminotransferase (ALT) and aspartate aminotransferase (AST), and diminishes the area of liver damage [19]. FXR is also required to promote liver repair after CCl_4-induced injury [21] and attenuates hepatic injury in a mouse model of alcoholic liver disease [22]. By regulating multiple intermediates in diverse metabolic pathways, FXR has become an attractive drug target for metabolic diseases.

Although some DHPs have been reported to interact with FXR by high-throughput screening [23–25], the binding mechanism and structure-activity relationship of these interactions are still unclear. In the present study, we resolved the binding mode of DHPs in FXR using crystallographic analyses and further confirmed the functional relationship between DHPs and the target FXR by in vivo mouse experiments. This research will not only explain the hepatoprotective effects of antihypertensive DHPs drugs, but also will reveal valuable clues for the design and development of next-generation Ca^{2+} blocker drugs with more hepatoprotective effects and provide a novel, safe structural template for the development of drugs targeting FXR.

2. Meterials and methods

2.1. Protein preparation

The human FXR ligand binding pocket (LBD) (residues 243–472) was expressed as an N-terminal 6 × His fusion protein from the expression vector pET24a (Novagen). BL21 (DE3) cells transformed with expression plasmids were grown in LB broth at 25 °C to an OD_{600} of ~1.0 and induced with 0.1 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG) at 16 °C. Cells were harvested and sonicated in 200 mL of extraction buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 10% glycerol and 25 mM imidazole) per 6 L of cells. The lysate was centrifuged at 20,000 rpm for 30 min, and the supernatant was loaded on a 5-mL $NiSO_4$-loaded HiTrap HP column (GE Healthcare). The column was washed with extraction buffer and the protein was eluted with a gradient of 25–500 mM imidazole. The FXR LBD was further purified on a Q-Sepharose column (Amersham Biosciences). A five-fold molar excess of compound was added to the purified protein, followed by filter concentration to 10 mg/mL to prepare the protein-ligand complex. The FXR LBD was complexed with a two-fold molar excess of the SRC2–3 peptide (QEPVSPKKKENALLRYLLDKDDTKD) before filter concentration.

2.2. Coregulator binding assays

The binding of the coregulator peptide motif to FXR LBD in response to ligands was determined by AlphaScreen assays using a hexahistidine detection kit from Perkin-Elmer as previously described [26]. DHPs were purchased from TargetMol (Shanghai, China). Experiments were conducted with approximately 20–40 nM FXR LBD and 20 nM biotinylated cofactor peptides in the presence of 5 μg/mL donor and acceptor beads in a buffer containing 50 mM MOPS, 50 mM NaF, 0.05 mM CHAPS, and 0.1 mg/mL bovine serum albumin, all adjusted to pH 7.4. The sequence of the N-terminal biotinylated SRC2–3 peptide is QEPVSPKKKENALLRYLLDKDDTKD.

2.3. Dual-luciferase reporter assay

HEK-293 T cells (ATCC) were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum and were transiently transfected using Lipofectamine 2000 (Invitrogen). All mutant FXR plasmids were created using the Quick-Change Site-Directed Mutagenesis Kit (Stratagene). Cells were plated in 24-well plates at a density of 5×10^4 cells per well; 24 h later, cells were co-transfected with plasmids encoding full-length FXR and the cognate luciferase reporter EcRE-Luc. Ligands were added 5 h after transfection. Cells were harvested 24 h later for luciferase assays with a dual-luciferase reporter assay system (Promega). The luciferase activities were normalized to the activity of a Renilla luciferase reporter construct that was co-transfected as an internal control.

2.4. Animals and treatments

11- to 12- week-old male mice were maintained under environmentally controlled conditions with free access to a standard chow diet and water. Animal experiments were conducted in the barrier facility of the Laboratory Animal Center, Xiamen University, and were approved by the Institutional Animal Use and Care Committee of Xiamen University, China. Wild-type (WT) and FXR knockout (KO) mice were treated with either vehicle (0.5% carboxymethyl cellulose sodium) or cilnidipine (5 mg/kg body weight) dissolved in vehicle by oral gavage once a day for five days. Six hours after the fifth treatment, 500 mg of APAP/kg body weight dissolved in PBS were i.p. injected into the mice, and the mice were sacrificed 24 h later. Liver tissue samples were collected to measure GSH levels (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The other liver tissues were collected for real-time quantitative reverse transcription PCR (RT-PCR). Sera were collected to measure enzyme activities, including LDH, ALT and AST, using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.5. H&E staining and TUNEL staining

Liver tissue samples were fixed with 4% paraformaldehyde, and the liver histology characterization was analysed by staining paraffin-embedded sections with haematoxylin and eosin (H&E) using standard procedures. Fluorescent transferase-mediated dUTP nick-end labeling (TUNEL) staining was performed using the DeadEnd Fluorometric TUNEL System kit (Promega). All sections were stained with DAPI and mounted in Vectashield (Vector Labs).

2.6. Crystallization and structure determination

The crystals of FXR/nicardipine complex were grown at room temperature in hanging drops containing 1.0 μL of the above protein-peptide solutions and 1.0 μL of well buffer containing 0.2 M NaSCN and 12% *w/v* Polyethylene glycol 3350, whereas the crystals of FXR/nimodipine were grown in the well buffer containing 0.2 M Magnesium chloride hexahydrate, 0.1 M Tris pH 8.5, and 25% *w/v* Polyethylene glycol 3350. The crystals of FXR/cilnidipine were obtained in the well buffer containing 2% Tacsimate pH 7.0, 0.1 M HEPES pH 7.5, and 20% *w/v* Polyethylene glycol 3350. The crystals of FXR/felodipine were obtained in the well buffer containing 0.2 M Lithium sulfate monohydrate, 0.1 M Tris pH 8.5, and 25% *w/v* Polyethylene glycol 3350. Crystals were directly flash-frozen in liquid nitrogen for data collection. The observed reflections were reduced, merged and scaled with DENZO and SCALEPACK in the HKL2000 package [27]. The structures were determined by molecular replacement using the CCP4 suite. Manual model building was performed with Coot [28], followed by Refmac5 refinement in the CCP4 suite.