EXPERIMENTAL CELL RESEARCH 327 (2014) 318–330

Available online at www.sciencedirect.com

ScienceDirect

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

Research Article

Ritonavir binds to and downregulates estrogen receptors: Molecular mechanism of promoting early atherosclerosis

Jin Xiang[a,1], *Ying Wang*[b,1], *Ke Su*[c], *Min Liu*[a], *Peng-Chao Hu*[b], *Tian Ma*[a], *Jia-Xi Li*[a], *Lei Wei*[b], *Zhongliang Zheng*[d,*], *Fang Yang*[e,*]

[a]*Ministry of Education Laboratory of Combinatorial Biosynthesis and Drug Discovery, School of Pharmaceutical Science, Wuhan University, Wuhan 430071, PR China*
[b]*Department of Pathophysiology, School of Medicine, Wuhan University, Wuhan 430071, PR China*
[c]*Department of Nephrology, Renmin Hospital of Wuhan University, Wuhan 430060, PR China*
[d]*State Key Laboratory of Virology, College of Life Sciences, Wuhan University, Wuhan 430072, PR China*
[e]*Department of Physiology, School of Medicine, Wuhan University, Wuhan 430071, PR China*

ARTICLE INFORMATION

Article Chronology:
Received 19 March 2014
Received in revised form
29 June 2014
Accepted 1 July 2014
Available online 10 July 2014

Keywords:
Ritonavir
Estrogenic actions
Estrogen receptor
Macrophage

ABSTRACT

Estrogenic actions are closely related to cardiovascular disease. Ritonavir (RTV), a human immunodeficiency virus (HIV) protease inhibitor, induces atherosclerosis in an estrogen-related manner. However, how RTV induce pathological phenotypes through estrogen pathway remains unclear. In this study, we found that RTV increases thickness of coronary artery walls of Sprague Dawley rats and plasma free fatty acids (FFA) levels. In addition, RTV could induce foam cell formation, downregulate both estrogen receptor α (ERα) and ERβ expression, upregulate G protein-coupled estrogen receptor (GPER) expression, and all of them could be partially blocked by 17β-estradiol (E2), suggesting RTV acts as an antagonist for E2. Computational modeling shows a similar interaction with ERα between RTV and 2-aryl indoles, which are highly subtype-selective ligands for ERα. We also found that RTV directly bound to ERα and selectively inhibited the nuclear localization of ERα, and residue Leu536 in the hydrophobic core of ligand binding domain (LBD) was essential for the interaction with RTV. In addition, RTV did not change the secondary structure of ERα-LBD like E2, which explained how ERα lost the capacity of nuclear translocation under the treatment of RTV. All of the evidences suggest that ritonavir acts as an antagonist for 17β-estradiol in regulating α subtype estrogen receptor function and early events of atherosclerosis.

Introduction

Ritonavir (RTV) is a human immunodeficiency virus (HIV) protease inhibitor (PI) used to provide immunologic benefits and improve the survival rates of HIV-infected individuals. However, more and more evidences have shown that RTV induces body fat changes and metabolic abnormalities, and many patients have developed premature atherosclerosis [1–3]. These side effects induced by PI have prevented the broad application of PIs. Thus, it is important to understand the mechanism by which PIs induce side effects.

*Corresponding authors.
E-mail addresses: biochem@whu.edu.cn (Z. Zheng), fang-yang@whu.edu.cn (F. Yang).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.yexcr.2014.07.002
0014-4827/

Interestingly, gender differences in atherosclerosis have been reported to be induced by PIs [2]. For example, estrogens and isoflavonoid phytoestrogens protect cardiovascular tissues from developing atherosclerotic lesions [2–4]. In addition, RTV reduce ethinyl estradiol level in blood by 40%, which is the major estrogen component in oral contraceptives [5]. These reports suggest that RTV may lead to atherosclerosis by impairing estrogenic actions.

Protein kinase C alpha-subtype (PKCα) expression was shown to inversely correlate with estrogen receptors (ER) expression in osteoblast-like cells and breast cancer [6–8], and the activation of PKCα highly correlated with cardiovascular disease [9,10], suggesting that PKCα may induce cardiovascular disease through inhibiting ER. In addition, our previous study showed that RTV induced foam cell formation by upregulating PKCα [11]. However, the question whether RTV regulates ER in macrophages still remains unresolved.

The biological actions of estrogen are largely mediated through two distinct ER isoforms, namely ERα and ERβ, which are widely distributed among tissues, including the cardiovascular system [12]. G protein-coupled estrogen receptor (GPER), also designated as G protein receptor 30 (GPR30), has been recently found to mediate the effects of estrogens in various normal and cancer cells. ERs function as ligand-activated transcription factors [13]. The ligand-binding domain (LBD) of ERs mediates ligand binding, receptor dimerization, nuclear translocation, and activation of target gene expression [14]. The following main factors can affect the estrogenic actions of the target cell: (1) estrogen levels in blood; (2) expression levels of ERs; (3) subcellular distribution of ERs; and (4) competitive antagonism of estrogen binding to ERs.

To gain insight into the role of RTV in the estrogen pathway and explore the mechanism by which RTV affects lipid metabolism and promotes early atherosclerosis formation, we have investigated these main factors that affect estrogenic actions in macrophages and the impacts of RTV on artery morphology, foam cell formation, and lipid levels in blood. Here, we demonstrated that RTV increased the thickness of the rat coronary artery wall and plasma free fatty acids (FFA) level without changing plasma estrogen levels. In addition, RTV induced foam cell formation and downregulates ERα and ERβ. We observed that RTV bound directly to ERα and had similar interactions to ERα as 2-aryl indoles, which are highly subtype-selective ligands for ERα [15]. We observed that RTV selectively inhibited the nuclear localization of ERα, and it acted as an antagonist for 17β-estradiol (E2) to regulate ER-induced gene. We observed that RTV did not change the secondary structure of ERα-LBD like E2, which explained how ERα lost the capacity of nuclear translocation under the treatment of RTV. We also observed that RTV upregulated the expression of GPER and inhibited the nuclear localization of GPER which was caused by E2.

Materials and methods

Animal and cell culture

All animal studies complied with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication no. 85-23, revised 1996). In addition, the study protocol involving animal use was approved by the China Hubei Provincial Science & Technology Department (SCXK 2003-0013). Male rats were used to avoid the cyclic hormonal changes in female rats that are associated with the estrus cycle and that could have confounded results [16]. RAW264.7, a mouse leukemic monocyte macrophage cell line, was obtained from China Center for Type Culture Collection (CCTCC) and cultured in phenol red-free-Minimum Essential Medium Eagle (MEM, Wuhan Boster, China) supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT, USA) under the conditions of 37 °C and 5% CO_2. The maximum concentration of RTV in plasma for clinical application was 15 μM, however, 15 and 30 μM RTV were chosen in the following cell studies for the reason described in our previous study [11].

Histology and hematoxylin and eosin (HE) staining

Twenty male Sprague Dawley rats (weighing 90–110 g upon arrival) were housed in groups of two in a temperature (23±2 °C) and humidity (50±5%) controlled animal facility. The rats were maintained under 12 h light/dark cycle with ad libitum access to food and water. For three weeks, male rats were orally administered with either a vehicle (5% ethanol: 95% propylene glycol) or RTV (125 mg/kg/day) and sacrificed on the 22nd day. Following drug treatment, blood collected by a phlebotomy was centrifuged at 1500 rpm for 15 min to separate plasma from red blood cells (RBCs). Plasma was immediately stored at −80 °C until further analysis. Rat coronary arteries were carefully collected and fixed in 4% buffered paraformaldehyde (PBS, pH 7.4) for HE staining. Tissue sections were then examined under microscopy (Canon PC 1099 Camera).

Estrogen and lipoprotein lipid analyses

Estradiol (E2) and testosterone (T) levels in plasma were measured by radioimmunoassay (RIA) [17]. Plasma FFA, total cholesterol (TC), high density lipoprotein (HDL), and triglyceride (TG) concentrations were measured in quadruplicate with enzymatic kits, standardized reagents, and standards using a VP Autoanalyzer (Abbott Laboratories, North Chicago, IL). The equation of Friedewald, Levy, and Fredrickson was used to calculate LDL-C concentrations [18].

Oil red O staining and microscopy assay

MEM containing 10% FBS was replaced with phenol red-free-MEM supplemented with 100 μg/mL LDL and plates were divided into four sections for different treatments: RTV (Mchem), phorbol-12-myristate-13-acetate (PMA, Sigma P8139), and E2 (Aladdin) were dissolved in DMSO and added to the culture medium to treat RAW264.7 cells. After incubation, the macrophage cells were fixed with 4% formaldehyde in PBS for 30 min followed by two washes with PBS. The cells were stained with 0.5% Oil Red O (Amresco) in 100% 1,2-propanediol for 30 min, washed three times with 85% 1,2-propanediol, and washed three times with distilled water; they were then examined by microscopy (Canon PC 1099 Camera).

Western blot analysis

After stimulating the macrophages with 15 and 30 μM RTV for 1 h, the cells were lysed in 200 μL ice-cold hypotonic buffer containing 150 mM NaCl, 1% Triton X-100, and 50 mM Tris–HCl (pH 8.0). The cells were incubated with 0.01, 0.1 and 1 μM E2 for 1 h to study the relationship between the activation of ER and expression levels of ERs in macrophages. The cells were incubated with 15 μM RTV plus