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ORIGINAL ARTICLE

Rilmenidine improves hepatic steatosis through p38-dependent pathway to higher the expression of farnesoid X receptor

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Abstract The nuclear receptor farnesoid X receptor (FXR) regulates pathways in lipid, glucose, and energy metabolism. Activation of FXR in mice significantly improved high-fat diet-induced hepatic steatosis. It has been reported that activation of imidazoline I-1 receptor by rilmenidine increases the expression of FXR in human hepatoma cell line, Hep G2 cell, to regulate the target genes relating to lipid metabolism; activation of FXR by rilmenidine exerts an antihyperlipidemic action. However, signals for this action of rilmenidine are still unknown. In the present study, hepatic steatosis induced in mice by high-fat diet was improved by rilmenidine after intraperitoneal injection at 1 mg/kg daily for 12 weeks. Also, mediation of I-1 receptors was identified using the specific antagonist efaroxan. Moreover, rilmenidine decreased the oleic acid-induced lipid accumulation in Hep G2 cells. Otherwise, rilmenidine increased the phosphorylation of p38 to increase the expression of FXR. Deletion of calcium ions by BAPTA-AM reversed the rilmenidine-induced p38 phosphorylation. In conclusion, we suggest that rilmenidine activates I-1 receptor to increase intracellular calcium ions that may enhance the phosphorylation of p38 to higher the expression of FXR for improvement of hepatic steatosis in both animals and cells.

Keywords Farnesoid X receptor · Hepatic steatosis · Imidazoline I-1 receptor · Rilmenidine · p38

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Introduction

Nonalcoholic fatty liver disease (NAFLD) is a chronic metabolic disorder characterized by lipid accumulation in the liver (Lewis and Mohanty 2010; Festi et al. 2004). The prevalence of NAFLD has been significantly increased recently and affects more than 25% of the general population and 80% of obese and/or diabetic patients (Szczepaniak et al. 2007; Wieckowska et al. 2007). NAFLD encompasses a broad spectrum ranging from steatosis, steatohepatitis, and to fibrosis (Tiniakos et al. 2010; Neuschwander-Tetri and Caldwell 2003). Risk of cardiovascular disease or diabetes was increased in patients with NAFLD (Soderberg et al. 2010; Targher et al. 2005). Therefore, investigation of effective therapeutic strategies for hepatic steatosis is important.

Modulation of orphan nuclear receptors and their downstream targets provides alternative therapeutic strategies for the treatment of lipid disorders. Farnesoid X receptor (FXR) belongs to one the nuclear hormone receptor superfamily, which is highly expressed in the liver and intestine (Forman et al. 1995; Lu et al. 2001; Zhang et al. 2003). FXR plays a critical role in blood lipid homeostasis through the control of cholesterol catabolism to bile acids (Maloney et al. 2000; Sinal et al. 2000; Edwards et al. 2002). Genes induced by FXR have been implicated in the maintenance of normal lipid level. FXR was discovered to serve as a sensor for sterol metabolism, which regulates the homeostasis of metabolism for glucose, cholesterol, or bile acids (Lu et al. 2001; Repa and Mangelsdorf 2002; Claudel et al. 2005). In addition, activation of FXR lowers plasma triglyceride level (Maloney et al. 2000; Kast et al. 2001) by a mechanism related to the decrease of hepatic SREBP-1c expression (Watanabe et al. 2004; Zhang et al. 2004). Also, FXR activation reverses insulin resistance and lipid abnormalities in addition to the protection against liver steatosis in Zucker (fa/fa) obese rats (Cipriani et al. 2010).

Activation of imidazoline receptors improves metabolic disorders. The imidazoline I-1 receptor mediates sympathoinhibitory actions to reduce plasma catecholamine levels (Ernsberger et al. 1997; Bousquet 2000). Also, activation of I-1 receptors by specific agonist rilmenidine significantly improved hypertension in obese spontaneously hypertensive rats named Koletsky rats (Velliquette et al. 2006). Activation of I-2 receptor increases plasma β-endorphin level to facilitate glucose uptake in the muscle for improvement of hyperglycemia (Lui et al. 2010). Activation of I-3 receptor stimulates insulin secretion from pancreatic β-cells to regulate blood glucose (Raasch et al. 2001). The recent study indicated that activation of I-1 receptor by rilmenidine regulates blood lipid through an increase of FXR expression (Niu et al. 2011). However, the potential signals for this increase of FXR by rilmenidine remained obscure.

In the present study, hepatic steatosis was induced in mice by high-fat diet (HFD) for identification of the role of imidazoline I-1 receptor in the improvement of hepatic steatosis by rilmenidine. Also, Hep G2 cells were used to investigate the potential mechanisms of rilmenidine-induced FXR expression.

Material and methods

Animals

C57BL/6J male mice were obtained from the animal center of National Cheng Kung University Medical College and fed with HFD containing 34.9% fat (wt./wt.) for 12 weeks (58Y1; TestDiet, Richmond, IN, USA) starting from 8 weeks of age as described in previous report (Zhang et al. 2010). Then, mice were divided into three groups. The first group was intraperitoneally (i.p.) injected with rilmenidine (Tocris Bioscience, Ellisville, MO, USA) at 1 mg/kg daily following our previous method (Niu et al. 2011). In the second group, 1 mg/kg efaroxan (Tocris Bioscience, Ellisville, MO, USA), one specific antagonist of imidazoline I-1 receptor, was also i.p. injected at 30 min before the injection of rilmenidine. Also, the third group received similar injection of the vehicle at same

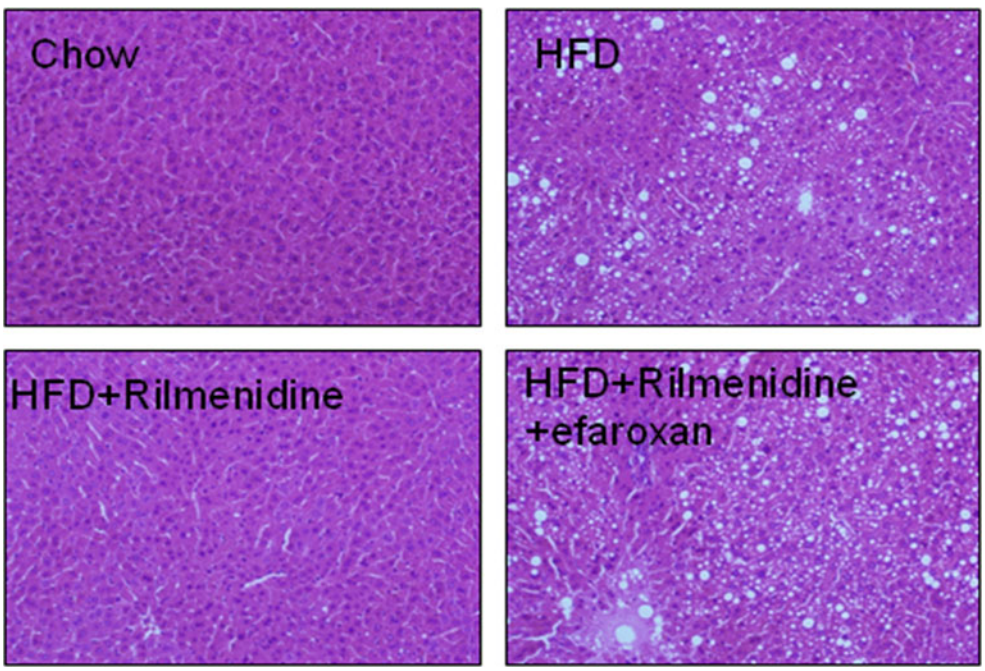

Fig. 1 Treatment of rilmenidine improves hepatic steatosis in high-fat diet-fed mice. The mice were fed with high-fat diet (*HFD*) for 12 weeks. One milligram per kilogram rilmenidine was daily i.p. injected into HFD-fed mice. One milligram per kilogram efaroxan was also i.p. injected into mice prior to the injection of rilmenidine for 30 min. At the end of experiment, livers were removed from mice for further investigation. Histology of liver was characterized by staining with H&E (magnification, ×400)

volume. The animal experiments were conducted in accordance with *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health, as well as the guidelines of Animal Welfare Act.

Histological analysis

The liver tissues were removed from each group of mice and fixed in 10% formaldehyde at 4°C for 2 days. Fixed specimens were dehydrated and embedded in paraffin. The specimens were then cut into 5-μm thick sections at 50-μm intervals and then stained with hematoxylin and eosin (H&E; Muto Pure Chemicals, Tokyo, Japan). The sections were then observed with a light microscope.

Cell cultures

Hep G2 cell line was purchased from Bioresource Collection and Research Center (Food Industry Research and Development Institute, Hsinchu City, Taiwan). The cells were maintained at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium (HyClone, South Logan, UT, USA) supplemented with 10% fetal bovine serum. The mitogen-activated protein kinases (MAPK) inhibitors SB203580, PD98059, and SP600125 or the calcium chelator BAPTA-AM were purchased from Tocris Bioscience for pretreatment at 30 min before the incubation of rilmenidine. For the induction of steatosis, the cells were starved in serum-free medium overnight. Stock solutions of 100 mM oleic acid (OA, Sigma-Aldrich) prepared in ethanol were diluted in culture medium containing 0.5% bovine serum albumin (BSA, Sigma-Aldrich) to obtain the desired final concentrations. Control cells were treated with OA-free medium containing 0.5% BSA. Staining of intracellular neutral lipids was performed with oil red O (Sigma-Aldrich).

Western blotting analysis

Total protein lysates from tissues or cells were extracted in lysis buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris (pH 7.5), 5 mM ethylenediaminetetraacetic acid) containing protease and phosphatase inhibitor cocktail (Sigma-Aldrich). The protein concentration was determined by BCA assay kit (Pierce Biotechnology, Rockford, IL, USA). Protein lysates (50 μg) were separated using 10% SDS–polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). The membrane was blocked at 25°C for 1 h in TBS-T [10 mM Tris (pH 7.6), 150 mM NaCl, and 0.05% Tween 20], containing 3% BSA, and probed with 1:1,000 primary antibodies, such as FXR (Santa Cruz Biotechnology, Santa Cruz, CA, USA), phospho-p38 MAPK (Thr180/Tyr182), phospho-ERK1/2 (Thr202/Tyr204), phospho-JNK (Thr183/Tyr185; Cell Signaling Technology, Beverly, MA, USA), and actin (Millipore) at 4°C overnight. After the membrane had been washed with TBS-T, the blots were incubated with a 1/5,000 dilution of horseradish peroxidase-conjugated secondary antibodies at 25°C for 1 h. The protein bands were visualized using enhanced chemiluminescence kit (PerkinElmer, Boston, MA, USA). Actin was an internal control. The optical densities of the bands were determined using software (Gel-Pro Analyzer 4.0; Media Cybernetics Inc., Silver Spring, MD, USA).

Statistical analysis

Data are expressed as the mean±SE for the number (n) of animals in the group as indicated. Repeated measures

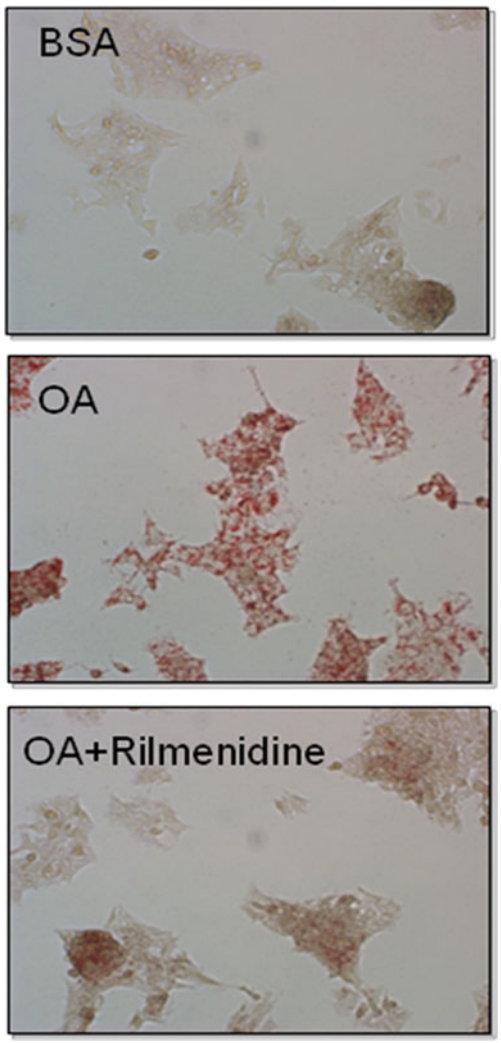

Fig. 2 Rilmenidine decreases oleic acid-induced lipid accumulation in Hep G2 cells. Prior to the treatment of 0.5 mM oleic acid (*OA*) for 6 h, Hep G2 cells were treated with 1 μM rilmenidine for 30 min. Otherwise, cells incubated with BSA were taken as control. The cells were then fixed with 4% paraformaldehyde and stain with oil red O to determine the accumulation of lipid

analysis of variance and Dunnett range post hoc comparisons were used to determine the source of differences where appropriate. Also, Bonferroni's correction was applied to the data which were obtained from relatively small groups. Significance is declared when P value is less than 0.05.

Results

Administration of rilmenidine into HFD mice improved hepatic steatosis through I-1 receptor

Mice fed with HFD significantly induced hepatic steatosis. Lipid droplets were observed in HFD mice. Administration of 1 mg/kg rilmenidine improved HFD-induced hepatic steatosis markedly. Role of imidazoline I-1 receptor in this rilmendine-improved hepatic steatosis was identified by exfaroxan, I-1 receptor specific antagonist. Pretreatment of exfaroxan significantly reversed rilmenidine-improved hepatic steatosis (Fig. 1).

Rilmenidine decreased lipid accumulation in oleic acid-treated Hep G2 cells

Hep G2 cell line was used to mimic the in vitro hepatic steatosis. Although Hep G2 cells are hard to show the same as normal human hepatocytes totally, this cell line is introduced to offer an alternative and reliable model for studies on liver lipid metabolism (Wang et al. 1988). Similar to the changes in primary culture of mouse hepatocytes (Niu et al. 2011), oleic acid-induced lipid accumulation in Hep G2 cells that also improved by rilmenidine (Fig. 2).

Rilmenidine activated P38 to induce the expression of FXR

The role of MAPK in rilmenidine-induced FXR expression was investigated in Hep G2 cells. Incubation of rilmenidine with Hep G2 cells significantly increased the phosphorylation of MAP kinases, including p38, ERK1/2, and JNK (Fig. 3a). Then, MAPK inhibitors were applied. SB203580 at concentration enough to inhibit p38 phosphorylation significantly reversed rilmenidine-induced FXR expression, whereas PD98059 (ERK1/2 inhibitor) and SP600125 (JNK inhibitor) failed to modify the effect of rilmenidine on FXR expression (Fig. 3b). The mediation of p38 in rilmenidine-induced FXR expression can thus be considered (Fig. 3).

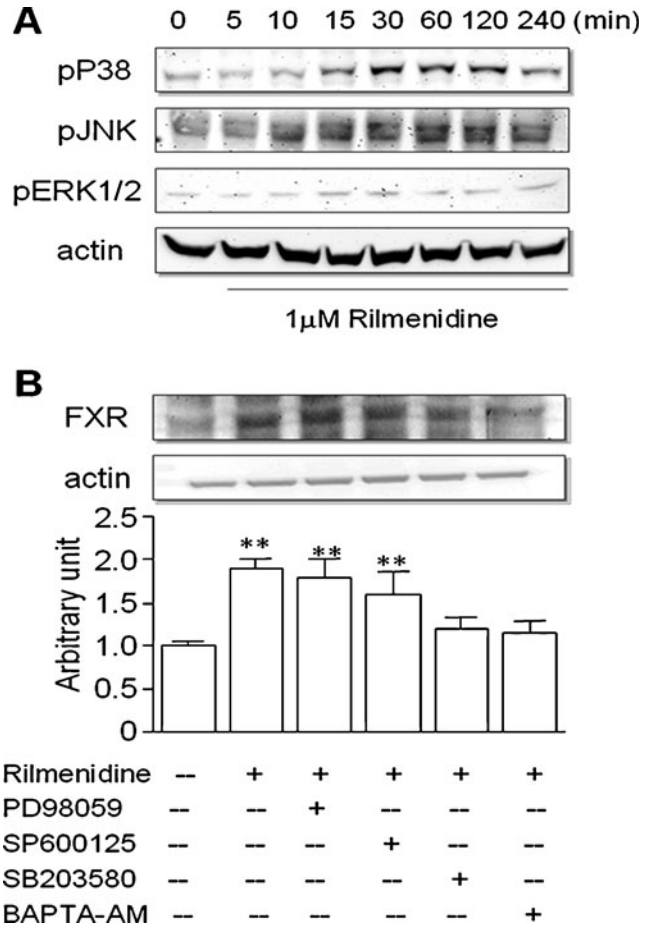

Fig. 3 Changes of MAPK phosphorylation by rilmenidine. **a** The cells were treated with 1 μM rilmenidine, then harvested at indicated times for the detection of MAPK phosphorylation. **b** The cells were pretreated for 30 min with 25 μM SB203580, PD98059, or SP600125, before the 3-h incubation of rilmenidine. Protein expression was detected by western blotting analysis. The cells were then harvested for the detection of MAPK phosphorylation. The protein levels were determined based on one representative experiment. Data are presented as the FXR to actin ratio displayed by a sample and are expressed as the means±SE of results from three experiments. $^{**}P<0.01$ as compared with control group

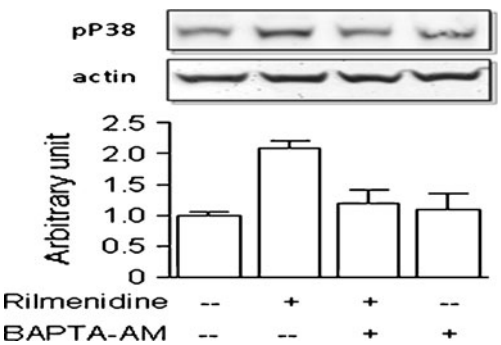

Fig. 4 The effect of BAPTA-AM on p38 phosphorylation. The cells were treated for 30 min with 25 μM BAPTA-AM before rilmenidine treatment for 15 min. Protein expression was detected by western blotting analysis. The protein levels were determined based on one representative experiment. Data are presented as the phospho-p38 to actin ratio displayed by a sample and are expressed as the means±SE of the result from three experiments. $^{**}P<0.01$ as compared with control group

Rilmenidine activated P38 though a calcium-dependent pathway

In order to know the upstream for rilmenidine-induced p38 phosphorylation, we used BAPTA-AM to bind with calcium ions. Pretreatment of BAPTA-AM significantly reversed the rilmenidine-induced p38 phosphorylation, while BAPTA-AM itself showed no effect on p38 phosphorylation (Fig. 4). Thus, rilmenidine-induced p38 phosphorylation in a calcium-dependent manner can be considered.

Discussion

In the study, we found that an activation of imidazoline I-1 receptor by the specific agonist rilmenidine significantly improved high-fat diet-induced hepatic steatosis at the effective dose as described previously (Niu et al. 2011). Also, this action of rilmenidine was reversed by pretreatment of the I-1 receptor antagonist, implying the role of I-1 receptor in the regulation of lipid homeostasis. Significant lipid accumulation was also observed in Hep G2 cell line treated with oleic acid, and this in vitro model was markedly reduced by treatment of rilmenidine (Fig. 2). It has been indicated that activation of I-1 receptor increases the expression of FXR (Niu et al. 2011), while activation of FXR may improve hepatic steatosis (Claudel et al. 2005). In the previous study (Niu et al. 2011), rilmenidine dose dependently induced the increase of FXR expression in Hep G2 cells to lower plasma lipid. In the present study, rilmenidine at the same effective dose also improved hepatic steatosis (Fig. 1). However, direct evidence showing rilmenidine-improved hepatic steatosis is still not mentioned before.

Imidazoline I-1 receptor is known to be sensitive to guanidine and belongs to the G protein-coupled receptor family (Ernsberger et al. 1995). Stimulation of I-1 receptor increases the accumulation of second messenger diglycerides (DAG, Separovic et al. 1996). Increase of DAG further activates protein kinase C (PKC, Ernsberger et al. 1997). Phosphatidylcholine-selective phospholipase C (PC-PLC) is also coupled to I-1 receptor signaling in PC-12 cells for the vasodepressor action (Separovic et al. 1997). In the present study, the PC-PLC inhibitor (D609) and PKC inhibitor (chelethyrine) were used to evaluate the role of PC-PLC and PKC in rilmenidine-induced FXR expression. However, pretreatment of D609 or chelethyrine showed no effect on rilmenidine-induced FXR expression (data not shown). Thus, PC-PLC and/or PKC seem not mediated in the FXR expression increasing action of rilmenidine.

It has also been indicated that activation of I-1 receptor increases the phosphorylation of MAPK (Edwards et al. 2001; El-Mas et al. 2009). Activation of I-1 receptor by moxonidine increases the phosphorylations of ERK and JNK, along with PKC, showing this receptor may play a role in cell growth (Edwards et al. 2001). The effect of moxonidine on ERK activation was blocked by the I1-receptor antagonist efaroxan and by D609 (Edwards et al. 2001). Similar to the previous study, rilmenidine also increased the phosphorylation of MAPK in the same time course with peaks at 30 min (Fig. 3a). Also, pretreatment of p38 inhibitor reversed this rilmenidine-induced FXR expression, whereas ERK1/2 and JNK inhibitors showed no influence (Fig. 3b). A p38-dependent pathway involved in increased expression of FXR by rilmenidine through activation of I-1 receptor can thus be considered.

It has been indicated that rilmenidine has an ability to elevate cytosolic free calcium concentration in suspended cerebral astrocytes (Ozog et al. 1998). Also, calcium ameliorates obesity induced by high-fat diet (Sun et al. 2011). Thus, we investigated the role of calcium ions in rilmenidine-induced p38 phosphorylation. Pretreatment of BAPTA-AM at concentration sufficient to lower cellular calcium level significantly reversed the rilmenidine-induced p38 phosphorylation, indicating the involvement of calcium ions in rilmenidine-induced p38 phosphorylation for higher FXR expression. This result is consistent with the previous reports showing correlation of calcium with the p38 MAPK pathway (Wu et al. 2011; Sun et al. 2011).

Taken together, we suggest that activation of I-1 receptor by rilmenidine can increase FXR expression through calcium-related p38 phosphorylation to improve hepatic steatosis. Thus, activation of I-1 receptor might be a new strategy and rilmenidine might be a suitable candidate for the treatment of hepatic steatosis.

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Financial disclosure The authors have no financial and commercial conflicts of interest.

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