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Research Paper

Activation of PPARγ by a Natural Flavonoid Modulator, Apigenin Ameliorates Obesity-Related Inflammation Via Regulation of Macrophage Polarization

Xiujing Feng [a], Dan Weng [c], Feifei Zhou [a], Young D. Owen [d], Haohan Qin [a], Jingfa Zhao [a], WenYu [a], Yahong Huang [a], Jiajia Chen [a], Haijian Fu [e], Nanfei Yang [a], Dianhua Chen [a], Jianxin Li [e], Renxiang Tan [a], Pingping Shen [a,b,*]

[a] State Key Laboratory of Pharmaceutical Biotechnology, School of life Sciences, Nanjing University, Nanjing 210046, China
[b] MOE Key Laboratory of Model Animal for Disease Study, Model Animal Research Center, Nanjing Biomedical Research Institute, Nanjing Biomedical Research Institute, Nanjing University, Nanjing 210046, China
[c] Center for Molecular Metabolism, Nanjing University of Science and Technology, Nanjing 210094, China
[d] Graduate Medical Education, Virginia Mason Medical Center, Seattle, WA 98101, USA
[e] Key Lab of Analytical Chemistry for Life Science, School of Chemistry and Chemical Engineering, Nanjing University, Nanjing 210046, China

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ABSTRACT

PPARγ has emerged as a master regulator of macrophage polarization and is the molecular target of the thiazolidinedione drugs. Here we show that apigenin binds and activates PPARγ by acting as a modulator. Activation of PPARγ by apigenin blocks p65 translocation into nuclei through inhibition of p65/PPARγ complex translocation into nuclei, thereby decreasing NF-κB activation and favoringM2 macrophage polarization. In HFD and ob/ob mice, apigenin significantly reverses M1 macrophage into M2 and reduces the infiltration of inflammatory cells in liver and adipose tissues, as well as decreases the levels of pro-inflammatory cytokines, thereby alleviating inflammation. Strikingly, apigenin reduces liver and muscular steatosis, decreases the levels of ALT, AST, TC and TG, improving glucose resistance obviously. Unlike rosiglitazone, apigenin does not cause significant weight gain, osteoporosis et al. Our findings identify apigenin as a modulator of PPARγ and a potential lead compound for treatment of metabolic disorders.

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1. Introduction

Nutrient excess and adiposity leads to chronic low-grade inflammation, which is referred to as obesity-related inflammation (Xu et al., 2003b). Obesity-related inflammation acts as a key pathogenic link between obesity and obesity-associated metabolic disorders, including insulin resistance (Xu et al., 2003a), type 2 diabetes (Duncan et al., 2003) and cancer (Howe et al., 2013). Thus, resolving the inflammation is one potential strategy to treat metabolic syndromes. Thus far, several drugs, such as metformin (Dinarello, 2010) and thiazolidinedione have been proven to restrain low-grade inflammation and therefore to treat insulin-resistance and correlated physiological functional disorders. However, further efforts are needed to develop newer and safer therapeutics to ameliorate obesity-related inflammation and reverse metabolic disorders.

PPARγ, which belongs to the PPAR family of ligand-inducible transcription factors, has been well documented to play a central role in adipogenesis and low-grade inflammation. PPARγ is implicated in the regulation of immunological events, playing an important role in mediating the differentiation and activation of immune cells, as well as modifying cytokine expression patterns and cell fates, thereby remodeling the immune balance (Cipolletta et al., 2012). In particular, PPARγ has been recognized as a pivotal anti-inflammatory regulator in atherosclerosis primarily through regulating the differentiation and functional

Abbreviations: Api, apigenin; Rosi, rosiglitazone; HFD, high fat diet; ND, normal diet; IHC, Immunological Histological Chemistry; PPARγ, peroxisome proliferator activated receptor γ; Fizz1, found in inflammatory zone (FIZZ)1; Arg1, arginase; CD206, mannose receptor C type 1; TZD, thiazolidinedione; H&E, hematoxylin and eosin; AnV, Annexin V-FITC; PI, propidium iodide; ITC, isothermal titration calorimetry; ROS, reactive oxygen species; NO, nitric oxide; SPF, specific-pathogen-free; ALT, alanine aminotransferase; AST, aspartate aminotransferase; SEM, standard error of mean; EMSA, electrophoretic mobility shift assay; HFD, high fat diet; WAT, white adipose tissue; ATM, adipocyte tissue macrophages; NF-κB, nuclear factor kappa B; TC, total cholesterol; TG, triglycerides; ITTs, insulin tolerance tests; CT, computer tomography; SPPARMs, selective PPAR modulators; LBD, ligand binding domain; DBD, DNA binding domain.

* Corresponding author at: State Key Laboratory of Pharmaceutical Biotechnology, School of life Sciences, Nanjing University, Nanjing 210046, China.
E-mail address: ppshen@nju.edu.cn (P. Shen).

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polarization of macrophages (Bouhlel et al., 2007). Macrophages are heterogeneous and plastic, and there are at least two major macrophage populations: those in a predominantly M1-polarised pro-inflammatory state and those in a predominantly M2-polarised anti-inflammatory state (Chinetti-Gbaguidi and Staels, 2011). M1 cells are efficient producers of effector molecules (ROS and NO) and inflammatory cytokines (IL-1R, TNF, IL-6, etc.) and express typical phenotypic molecules, such as CD80 and CCR7. In contrast, the various forms of M2 cells share a distinct major signature with low IL-12, low IL-23, and high IL-10 and generally have high levels of scavenger, mannose, and galactose-type receptors, and arginine metabolism within these cells is shifted to production of ornithine and polyamines via arginase. M2 cells have been shown to express high levels of certain genes, such as chitinase-like Ym1, found in inflammatory zone (FIZZ)1 (Fizz1), arginase (Arg1) and mannose receptor C type 1 (CD206); these have become classical markers of M2 cells. M1 and M2 cells have distinct chemokine and chemokine receptor repertoires and therefore orchestrate different immune responses (Mantovani et al., 2004). Manipulation of M1/M2 homeostasis has been shown to be an effective strategy for clinical treatment of some inflammatory diseases. PPARγ activation can skew macrophages towards an anti-inflammatory M2 phenotype, resulting in inhibition of inflammation. Due to the role of PPARγ in macrophage polarization and anti-inflammation, PPARγ ligands have been used to treat metabolism-related inflammation and have shown significant anti-inflammatory therapeutic activity. Full agonist of PPARγ refers to a ligand which can bind to LBD domain with high-affinity and activate PPARγ thoroughly. For example, administration of pioglitazone, a full agonist of PPARγ, which can reduce the expression of IL-1β, IL-6, MCP-1, and TNF-α in peritoneal macrophages (Dasu et al., 2009), while rosiglitazone, another full agonist, upregulates the production of the anti-inflammatory molecule adiponectin, and thus decreases insulin resistance (Yang et al., 2002).

Ongoing work at developing ligands and modulators of PPARγ is focused on harnessing its anti-inflammatory properties. In recent years, some PPARγ agonists with anti–inflammatory effects (pioglitazone, Sitagliptin metformin/rosiglitazone combination) have already completed clinical trials. However, even with such promising therapeutic activity, the side effects of thiazolidinedione (TZD) drugs include cardiovascular failure, liver toxicity, bone fractures and potential carcinogenesis, these have greatly limited their clinical use (Lehrke and Lazar, 2005). Therefore, much attention has recently been paid to further optimization of the PPARγ ligands' structures to decrease or abrogate their side effects. In particular, exploration of natural compounds represents one promising strategy for developing new, safer ligands or modulators of PPARγ (Doshi et al., 2010). Apigenin (Api, 4,5,7-trihydroxyflavone) is a naturally occurring plant flavonoid abundant in various fruits and vegetables (Havsteen, 2002). It has lately gained attention as a beneficial and healthy compound because of its various biological effects and low intrinsic toxicity. Moreover, Api has been demonstrated to possess distinct anti-inflammatory activity in chronic inflammation (Choi et al., 2014) and skin inflammation (Byun et al., 2013). Api is also an inhibitor of NAD + ase CD38 and improves metabolic syndrome (Escande et al., 2013). In addition, Nicholas et al. has found that Api can specifically modulate NF-kB in macrophages by suppression the phosphorylation of p65 (Nicholas et al., 2007). It is noteworthy that Api might be a ligand of PPARγ via structure-based virtual screening (Salam et al., 2008a, Mueller et al., 2008). Hence, further study of Api and the underlying mechanisms related to the PPARγ pathway has potential therapeutic implications. In the current study, we identify Api as an effective ligand of PPARγ in macrophages. Importantly, Api can significantly attenuate obesity-related inflammation and metabolic disorders in high-fat diet-induced mice and ob/ob mice. Furthermore, unlike Rosi, a full ligand-type agonist of PPARγ, Api does not exhibit some adverse effects, such as obvious weight gain, osteoporosis, the increase of small adipocytes in white adipose tissue (WAT) and the accumulation of triglycerides in the serum of obese mice.

2. Materials and Methods Results

2.1. Reagents

Api (5,7-dihydroxyflavone, PubChem CID: 5281607, purity > 99%, chemical structure shown in Fig. 1a, purchased from Zelang biotechnology company (Nanjing, China) was dissolved in 100% DMSO. The final DMSO concentration in cell culture did not exceed 0.1% throughout the study. Rosi (PubChem CID: 77999) and GW9662 (PubChem CID 644213) were purchased from Sigma (St. Louis, MO). DMEM and RPMI1640 media were purchased from Gibco (Grand Island, NY). Penicillin and streptomycin, HRP-conjugated Goat Anti-Mouse IgG (H + L), FITC-coupled secondary antibody (be), Cy3-labeled Goat Anti-Mouse IgG (H + L), neutral red, CCK-8 and MTT are from Beyotime (Haimen, Jiangsu, China). TNF-α, IL-10, CCL2 and IL-1β ELISA assay kits were purchased from eBioscience (San Diego, CA). Alanine/aspartate aminotransferases (ALT/AST) assay kits, TC, TG, glucose and insulin kit were from Jiancheng Biology Institution (Nanjing, Jiangsu, China). Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA). PPRE-Luc plasmid and dual-luciferase reporter assay systems were from Promega (Madison, WI, USA).

2.2. Mice Treatment

Male C57BL/6J mice (3–4 weeks old) and male ob/ob mice were purchased from Animal Genetics Research Center of Nanjing University (Nanjing, China) and housed in a specific-pathogen-free (SPF) facility. Mice, starting at 3–4 weeks old, were randomly divided into four groups ($n = 9$ per group). Mice were fed for 16 weeks with either a normal chow diet (ND) consisting of 4.5% fat or a high-fat diet (HFD) (D12492, 60% fat, 20% carbohydrate, 20% protein, total 5.24 kcal/g; Research Diets Inc., New Brunswick, NJ). 19 weeks year-old HFD mice were grouped and injected with Api (10 mg/kg, 30 mg/kg or 50 mg/kg) (Liu et al., 2005, Dou et al., 2013), 10 mg/kg Rosi or vehicle alone (saline containing 0.1% DMSO) intraperitoneally daily for 21 days. Mice were weighed daily until sacrificed under anesthesia using diethylether. In addition, three month-old male C57BL/6J ob/ob mice were injected with 30 mg/kg Api or vehicle alone (saline containing 0.1% DMSO) intraperitoneally daily for 21 days. Animal welfare and experimental procedures were followed in accordance with the Guide for Care and Use of Laboratory Animals (National Institutes of Health, the United States) and the related ethical regulations of Nanjing University.

2.3. Isolation and Purification of Peritoneal Macrophages

Normal resident peritoneal cells of the Male C57BL/6J mice treated with Api (10 mg/kg, 30 mg/kg or 50 mg/kg), 10 mg/kg Rosi or vehicle alone for 21 days were obtained by peritoneal washing with 20 mL Dulbecco's PBS (D-PBS) containing 2% FBS and 40 mg/mL gentamicin. After centrifugation at 350g for 5 min, red blood cells were lysed in ACK buffer and mononuclear cells were re-suspended in complete medium (DMEM, 10% FBS, 2 mM L-glutamine, 100 units/mL penicillin and 100 units/mL streptomycin) and incubated at 37 °C for 2 h in plastic culture plates. Then, the non-adherent cells were removed, and the adherent cells were cultured in complete medium with different stimuli.

2.4. Isolation and Purification of Adipocyte Tissue Macrophages

Epididymis fat was excised and minced in Hanks' Balanced Salt Solution (HBSS; Invitrogen) containing calcium, magnesium and 0.5% BSA. Collagenase (Type II; Sigma-Aldrich, St Louis, MO) was added to a final concentration of 1 mg/mL and tissue suspensions were incubated at 37 °C for 20–30 min with constant shaking. The resulting cell suspensions were filtered through a 100-μm filter and centrifuged at 500g for 10 min to separate floating adipocytes from the SVC-containing pellet.

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