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Bixin regulates mRNA expression involved in adipogenesis and enhances insulin sensitivity in 3T3-L1 adipocytes through PPARγ activation

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ABSTRACT

Insulin resistance is partly due to suppression of insulin-induced glucose uptake into adipocytes. The uptake is dependent on adipocyte differentiation, which is controlled at mRNA transcription level. The peroxisome proliferator-activated receptor (PPAR), a ligand-regulated nuclear receptor, is involved in the differentiation. Many food-derived compounds serve as ligands to activate or inactivate PPAR. In this study, we demonstrated that bixin and norbixin (annatto extracts) activate PPARγ by luciferase reporter assay using GAL4–PPAR chimera proteins. To examine the effects of bixin on adipocytes, 3T3-L1 adipocytes were treated with bixin or norbixin. The treatment induced mRNA expression of PPARγ target genes such as adipocyte-specific fatty acid-binding protein (aP2), lipoprotein lipase (LPL), and adiponectin in differentiated 3T3-L1 adipocytes and enhanced insulin-dependent glucose uptake. The observations indicate that bixin acts as an agonist of PPARγ and enhances insulin sensitivity in 3T3-L1 adipocytes, suggesting that bixin is a valuable food-derived compound as a PPAR ligand to regulate lipid metabolism and to ameliorate metabolic syndrome.

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Introduction

Adipocyte differentiation is a complex process regulated by various transcriptional factors such as CCAAT/enhancer-binding proteins (C/EBPs) and cAMP-response element-binding protein (CREB). Peroxisome proliferator-activated receptor-γ (PPARγ) is a nuclear receptor, which is regulated by small hydrophobic compounds [1]. PPARγ activation is essential for the adipocyte differentiation process because PPARγ knock-out causes drastic suppression of the process [2–4]. Thus, various PPARγ ligands promote the adipocyte differentiation. The promotion of adipocyte differentiation results in increase of the number of insulin sensitive cells so that systemic insulin sensitivity is increased. Therefore, the synthetic ligands for PPARγ have been widely used as effective drugs for the treatment of type-2 diabetes caused by insulin resistance [5]. In this sense, appropriate spatial and temporal controls of the ligand-dependent PPARγ activation are necessary for not only physiological maintenance of energy storage but also improvement of insulin resistance.

The extract of a natural pigment annatto, which is extensively used in many processed foods, shows various bioactive properties including antioxidation and anti-tumor proliferation [6,7]. Bixin and norbixin (a water soluble and hydrolyzed derivative of bixin) have been found to be the main components of the extract and identified as the carotenoids having antioxidative and anticarcinogenic effects [8–11]. Bixin is unique among the naturally occurring carotenoids because of its two carboxylic groups, one of which is a methyl ester. Human plasma bixin and norbixin concentrations after uptake reach significant levels and their complete plasma clearance generally occurs after 8 h for bixin and after 24 h for norbixin [12]. Thus, it is reasonable to consider that bixin in processed foods may be an important nutritional factor for human health. However, the effects of bixin and norbixin on diabetic conditions have not been completely elucidated.

In this study, to examine the effects of bixin on adipocytes, differentiated 3T3-L1 adipocytes were treated with bixin and norbixin. These compounds induced the expression of PPARγ target genes in 3T3-L1 adipocytes such as adiponectin. It was also shown by luciferase assay using GAL4–PPAR chimera proteins that bixin activated PPARγ. Moreover, bixin and norbixin enhanced insulin-dependent glucose uptake into differentiated 3T3-L1 adipocytes. These findings indicate that bixin and norbixin induce the PPARγ

Abbreviations: PPAR, peroxisome proliferator-activated receptor; TG, triacylglyceride; DBD, DNA-binding domain; LBD, ligand-binding domain; MM, maintenance medium; DM, differentiation medium; LPL, lipoprotein lipase; 2DG, 2-deoxyglucose

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activation in 3T3-L1 adipocytes to stimulate insulin-dependent glucose uptake, suggesting that bixin and norbixin are food-derived compounds useful for improving and preventing insulin resistance.

Materials and methods

Chemicals and cell culture. Bixin and norbixin were purchased from Nacalai Tesque (Kyoto, Japan). All other chemicals were from Sigma (MO, USA) or Nacalai Tesque. Monkey CV1 kidney cells and mouse 3T3-L1 preadipocytes were purchased from American Type Culture Collection. All the cell lines were maintained in a maintenance medium (MM; 10% fetal bovine serum, 200 μM ascorbic acid, and 10 mg/ml penicillin/streptomycin in Dulbecco's modified Eagle's medium) at 37 °C in 5% CO_2/95% air under a humidified condition. For adipocyte differentiation, 2 days after reaching confluence, 3T3-L1 preadipocytes were incubated in a differentiation medium (DM), which was the maintenance medium supplemented with 0.25 μM dexamethazone, 10 μg/ml insulin, and 0.5 mM 3-isobutyl-1-methylxanthine, as described previously [13,14]. After 40 h, the cell culture medium was changed to a post-DM, which was MM supplemented with 5 μg/ml insulin, and then the medium was changed with a fresh one every 2 days. The samples for biochemical assays were prepared using cells cultured on 6-well tissue culture plates. Oil-Red O staining was performed as previously described [14]. The contents of cellular triacylglyceride and medium free glucose were measured by using TG Test WAKO Kit and AG Test WAKO Kit (Wako Pure Chemical Ind. Ltd., Osaka, Japan). Protein concentrations of samples for immunoblotting were defined by using Protein Assay Kit (Bio-Rad, CA, USA). Cell viability was measured by MTT assay as previously described [21].

Luciferase assays. Luciferase assay was performed as previously described [15–17]. Briefly, for luciferase assay using the GAL4–chimera protein, we transfected the following three plasmid DNAs into CV1 cells cultured on 24-well tissue culture plates; p4xUASg-tk-luc (a reporter plasmid), pM-PPARγ or pM-PPARα (an expression plasmid for the GAL4/PPAR ligand-binding domain chimera protein), and pRL-CMV (the internal control). The transfection was performed using LipofectAMINE. Twenty-four hours after the transfection, the transfected cells were cultured in MM containing bixin or norbixin for another 24 h. Luciferase assay was performed using the dual luciferase system (Promega, MO, USA) in accordance with the manufacturer's protocol.

RNA preparation and quantification of gene expressions. RNA samples of differentiated 3T3-L1 adipocytes were prepared using cells cultured on 6-well tissue culture plates 8 days after differentiation induction. The cells were treated with MM containing the PPARγ ligand for 24 h before RNA sample preparation as previously described [18–20]. Total RNA samples were prepared from cells cultured on 6-well tissue culture plates and mouse fat tissues using Sepasol Super-I (Nacalai Tesque) and SV total RNA isolation system (Promega), respectively, in accordance with each manufacturer's protocol. Aliquots of total RNA were reverse-transcribed using M-MLV reverse transcriptase (Invitrogen Corp.) in accordance with the manufacturer's instructions using a thermal cycler (Takara PCR Thermal Cycler SP: Takara Shuzo Co., Kyoto, Japan). To quantify mRNA expression, PCR was performed using a fluorescence temperature cycler (LightCycler System: Roche Diagnostics, Mannheim, Germany), as described previously [13,14]. All primer sets used in this study were described in our previous reports [13,15]. To compare mRNA expression level among samples, the copy number of each transcript was divided by that of GAPDH showing a constant expression. mRNA expression level is presented as the ratio compared with a control in each experiment.

[^{3}H]-labeled 2-deoxy-glucose uptake assay. Differentiated 3T3-L1 adipocytes (10 days after the differentiation induction) were cultured on 24-well plates. The cells were incubated in serum free-DMEM for 5 h. After washing with HKR buffer (containing 140 mM NaCl, 5.0 mM KCl, 1 mM $CaCl_2$, 2.5 mM $MgSO_4$, and 20 mM Hepes; pH 7.4), the cells were incubated with 0.5 ml of HKR buffer with 1% BSA at 37 °C for 20 min in the presence or absence of 5 μg/ml insulin. [^{3}H]-2-Deoxy-glucose (American Radiolabeled Chemicals, Inc., MO, USA) was added to a final concentration of 0.1 mM and incubated for 10 min. The cells were washed with cold PBS and solubilized in 0.1 N NaOH. The radioactivity of a 200 μl aliquot was determined using a scintillation counter (LS6500, Beckman Coulter, Inc., CA, USA). Glucose uptake was normalized to protein concentration.

Statistical analysis. The data are presented as means ± SEM and were statistically analyzed by the unpaired *t*-test or the Welch *t*-test when variances were heterogeneous. Differences were considered significant when P was <0.05.

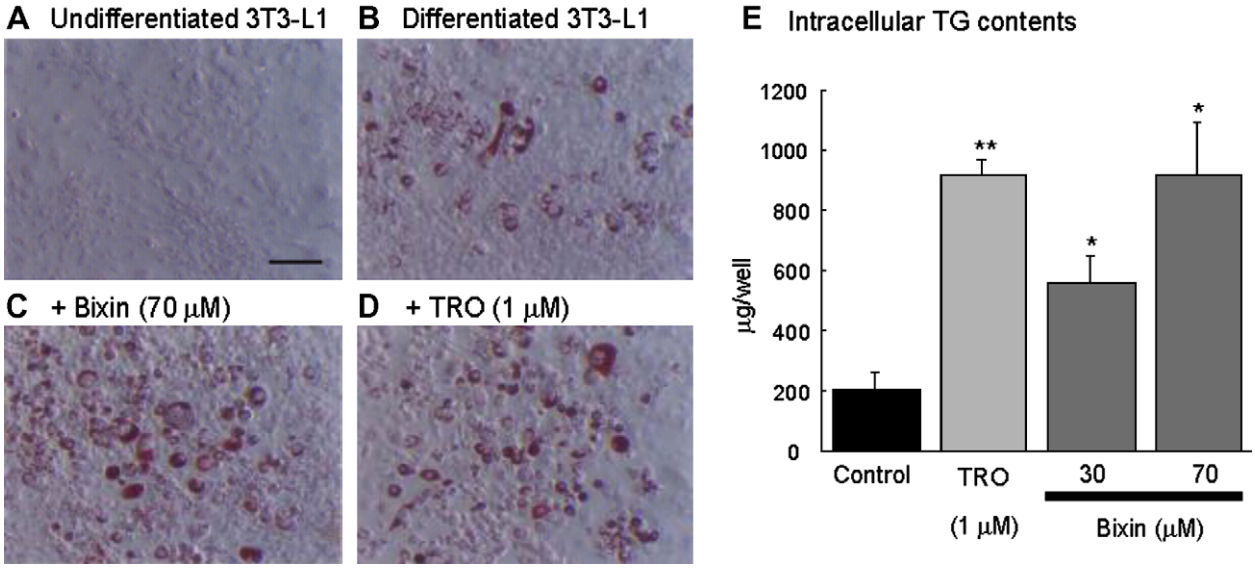

Fig. 1. Effects of bixin on adipocyte differentiation. (A–D) Photographs of representative bixin- or troglitazone (TRO)-treated 3T3-L1 adipocytes are shown in Oil-Red O stained. The bar in (A) indicates 50 μm. (E) At the same point, the cellular triacylglyceride (TG) content was measured. Control is the differentiated 3T3-L1 adipocytes shown in (B). Results are shown as means ± SEM. $^{*}P < 0.05$ and $^{**}P < 0.01$, compared with control.

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