1) Running title: Isorhamnetin activates lysosomes in J774.1 macrophages.

2) title: Isorhamnetin, a 3'-methoxylated flavonol, enhances the lysosomal proteolysis in J774.1 murine macrophages in a TFEB-independent manner.

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6) Abbreviations:

ANOVA, analysis of variance; ApoE, apolipoprotein E; ATP6V0D2, ATPase H^+ transporting V0 subunit d2; BAF, bafilomycin A1; BODIPY, boron dipyrromethene; BSA, bovine serum albumin; CTSD, cathepsin D; CTSF, cathepsin F; DMEM, Dulbecco's modified eagle medium; DMSO, dimethyl sulfoxide; EGCG, epigallocatechin-3-gallate; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPLC, high-performance liquid chromatography; LAMP1, lysosomal-associated membrane protein 1; LAMP2A, lysosomal-associated membrane protein 2A; LC-MS/MS, liquid chromatography tandem mass spectrometry; MITF, microphthalmia-associated transcription factor; MRM, multiple reaction monitoring; mTORC1, mechanistic target of rapamycin complex 1; PBS, phosphate-buffered saline; PPARγ, peroxisome

proliferator-activated receptor γ; RT-qPCR, reverse transcription quantitative polymerase chain reaction; SDS, sodium dodecyl sulfate; SNARE, soluble N-ethylmaleimide-sensitive-factor attachment protein receptor; TBS, Tris-buffered saline; TFA, trifluoroacetic acid; TFE3, transcription factor binding to IGHM enhancer 3; TFEB, transcriptional factor EB; TFEC, transcription factor EC; V-ATPase, vacuolar-type proton ATPase

Abstract

Lysosome is the principal organelle for the ultimate degradation of cellular macromolecules, which are delivered through endocytosis, phagocytosis and autophagy. The lysosomal functions have been found to be impaired by fatty foods and aging, and more importantly, the lysosomal dysfunction in macrophages has been reported as a risk of atherosclerosis development. In this study, we searched for dietary polyphenols which possess the activity for enhancing the lysosomal degradation in J774.1, a murine macrophage-like cell line. Screening test utilizing DQ-BSA digestion identified isorhamnetin (3'-*O*-methylquercetin) as an active compound. Interestingly, structural comparison to inactive flavonols revealed that the chemical structure of the B-ring moiety in isorhamnetin is the primary determinant of its lysosome-enhancing activity. Unexpectedly isorhamnetin failed to inhibit mTORC1-TFEB signaling, a master regulator of lysosomal biogenesis and function. Our data suggested that the other molecular mechanism might be critical for the regulation of lysosomes in macrophages.

(145/150 words)

Key words

isorhamnetin, lysosome, polyphenol, macrophage, TFEB

Introduction

The lysosome is an acidic organelle which plays the pivotal role in the degradation of extracellular or intracellular macromolecules. More than 60 kinds of acid hydrolases are identified to function inside lysosomes, which enables to digest a broad range of substrates into catabolites such as amino acids, fatty acids and monosaccharides. Also, it is well known that various kinds of characteristic proteins, including structural proteins like lysosomal-associated membrane protein 1 (LAMP1) family, vacuolar-type proton ATPase (V-ATPase) complexes for the lysosomal acidification, the lysosomal proteases (cathepsins), and soluble N-ethylmaleimide-sensitive-factor attachment protein receptor (SNAREs) that mediate its fusion with other organelles, are essential for the lysosomal functions. [1, 2]

Many reports have demonstrated that the lysosomal dysfunction is associated with the onsets of several diseases. Loss-of-function mutations in lysosomal hydrolases or lysosomal membrane proteins such as β-galactosidase and mucolipin-1 have been shown to lead to the intracellular accumulation of aberrant lysosomes, which causes Fabry disease and mucolipidosis type IV, respectively [3, 4, 5]. Importantly, beside these genetic mutation or deletion, recent reports suggested the possibility that aging and chronic high-fat diet might also attenuate lysosomal proteolysis activity [6, 7, 8].

Macrophages are professional phagocytic cells which engulf and digest apoptotic cells and exogenous bacteria for the immune response. It is obvious that adequate lysosomal activity is indispensable for macrophages to accomplish the phagocytosis process. Indeed, it has been reported that the genetic deletion of glucocerebrosidase, a lysosomal glycoside hydrolase, impaired endocytosis in macrophages and increased susceptibility to tuberculosis [9]. Furthermore, Sergin *et al.*

found that augmentation of lysosomal activity in macrophages by overexpressing transcriptional factor EB (TFEB), a master regulator of lysosomal biogenesis and function, suppressed the development of aortic root atherosclerosis in apolipoprotein E (ApoE) -knockout mice [10]. These observations suggested that the maintenance of lysosomal function in macrophages might be a potential therapeutic target for these diseases.

Polyphenols are plant-derived ingredients with multiple phenol structural units, and we intake them from vegetables and fruits in our daily diet. They are classified into flavonoids, lignans, stilbenes and curcuminoids according to the differences in chemical structures. In particular, flavonoids have been well-studied about their functionality and further classified into nine chemical groups (flavones, flavonols, isoflavones, flavans, flavanols, flavanones, flavanonols, chalcones, and anthocyanidins) [11]. Although it is noted that several flavonoids, such as quercetin, kaempferol and epigallocatechin-3-gallate (EGCG), have been reported to enhance autophagy [12, 13, 14] in some *in vitro* models, few studies have focused on the lysosomal proteolysis activity in macrophages.

Recent studies have identified TFEB as one of the most critical regulators of lysosomal numbers and activities. Under the nutrients-rich condition, TFEB is negatively regulated by mechanistic target of rapamycin complex 1 (mTORC1) in the cytoplasm [15], whereas amino acids depletion immediately induce TFEB transactivation through mTORC1 inactivation, followed by the upregulation of global gene expressions involved in lysosomal biogenesis and function [16]. Consistent with these studies, Moskot *et al*. showed that the treatment of genistein (4′, 5, 7-trihydroxyisoflavone) upregulated some lysosomal gene expressions such as cathepsin F (*Ctsf*) and mucolipin 1 (*Mcoln1*), through TFEB transactivation in Hela cells [17].

In this study, we screened for polyphenol compounds which enhance lysosomal

proteolysis in a macrophage-like cell line, J774.1. We then found that the treatment of isorhamnetin (3'-*O*-methylquercetin) significantly activated the lysosomal proteolysis. Additionally, it was also interesting that the chemical structure of B-ring in isorhamnetin were significant for its bioactivity. The potential molecular mechanism of isorhamnetin activity was investigated, which was suggested to be independent on mTORC1-TFEB signaling.

Materials and Methods

Reagents

Dulbecco's modified eagle medium (DMEM, 4.5 g/L glucose, liquid; 08458-16), penicillin-streptomycin mixed solution (Stabilized; 09367-34), protease inhibitor cocktail, phosphatase inhibitor cocktail (04080-11), and bovine serum albumin (BSA; 01281-26) were obtained from Nacalai Tesque Inc. (Kyoto, Japan). Pronase protease (Streptomyces griseus; 9036-06-0) was from Sigma-Aldrich (St. Louis, MO, USA). HRP-conjugated antibodies to mouse or rabbit IgG (AP124P or AP124P) were purchased from Millipore Inc. (Billerica, MA, USA). Rabbit polyclonal antibody to Phospho-4E-BP1 (Thr37/46) (236B4; #7547) was from Cell Signaling Technology Inc. (Beverly, MA, USA). Mouse polyclonal antibody to β-Actin (C4; sc-4778) was purchased from Santa Cruz Biotechnology Inc. (Dallas, TX, USA). DQ™ Green BSA (D12050) was obtained from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum (FBS; 10270-106) and FluoroBrite™ DMEM (A1896701) was from Thermo Scientific Inc. (Waltham, MA, USA). Xfect™ Transfection Reagent (631317) was purchased from Takara Inc. (Kyoto, Japan). Hoechst 33342 (CDX-B0030) was obtained from Chemodex Inc. (St. Gallen, Switzerland). Methanol (25183-70) and acetonitrile (01031-70) were from Kanto

Chemical Co. Inc. (Tokyo, Japan). Actinomycin D (014-21261) was purchased from FUJIFILM Wako Pure Chemical Co. (Tokyo, Japan). Bafilomycin A1 (14005) and Torin1 (10997) were obtained from Cayman Chemical Co. (Ann Arbor, MI, USA). Polyphenol compounds are obtained by the way shown below. 3'-*O*-Me-luteolin (1104S), 6-*O*-Me-luteolin (520-11-6), 7,4'-dihydroxyflavone (1259), datiscetin (1141S), eriodictyol (0056), flavonol (1026), homoeriodictyol (1118S), isoramnetin-3G (1228), isorhamnetin (1120S), kaempferol-3G (1243G), luteolin-7G (1126S), Q3G (0074), quercetagetin (1030), rhamnetin (1136S), tamarixetin (1140S) and tricetin (1335S) were from Extrasynthese (Genay, France). 2,2',4'-trihydroxychalcone (T502), 4,2',5'-trihydroxychalcone (22-314) and gossypetin (G500) were from INDOFINE Chemical Company Inc. (NJ, USA). Flavone (16012-31), morin (23416-31) and pyrogallol (29703-52) were from Nacalai Tesque Inc. (Kyoto, Japan). EGCG (E4268), hyperoside (00180585-25MG) and phloroglucinol (79330-25G) were from Sigma-Aldrich (MO, USA). 1,2,4-trihydroxybenzene, daidzin, piceid, rutin and tangeretin were from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). 3,4-dihydroxybenzoic acid, 3,5-dihydroxybenzoic acid, cyanidin, hesperetin and sudachitin were from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Other polyphenols were from natural compound libraries (S990043-NAT1/NAT2) purchased from Sigma-Aldrich (MO, USA).

Cell culture

J774.1, mouse macrophage-like cell line, was obtained from American Type Culture Collection. RAW264.7, mouse macrophage-like cell line, was kindly provided by Dr. T. Nikawa (Tokushima University). They were cultured in DMEM with 10% FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37°C under humidified 5% CO_2

atmosphere.

DQ-BSA degradation assay

DQ^{TM} Green BSA (DQ-BSA) was dissolved in phosphate-buffered saline (PBS) to make 1 mg/mL solution. Polyphenol compounds were dissolved in dimethyl sulfoxide (DMSO) to make 10 mM solution. For measurement of degradation products of DQ-BSA in cells, cells were seeded on black-wall clear-bottom 96-well plates (655090; Greiner Bio-One, Frickenhausen, Germany) (6.0×10^4 cells/well) and pre-cultured overnight. They were treated each polyphenol for indicated time with a final DMSO concentration of 0.1%, then added the DQ-BSA solution to a final concentration of 10 μg/mL. After incubation for 1 h, to quantify fluorescence intensity from digested DQ-BSA, cells were imaged by using Operetta high-content imaging system (PerkinElmer, MA, USA) at 40x magnification at following settings: (λ ex: 460-490 nm, λ em: 500-550 nm). Cell shapes were individually recognized by digital phase contrast and fluorescent intensity in each cell was quantified using dedicated imaging software (Harmony 4.5; PerkinElmer, MA, USA). In the case of using a fluorescent scanner, cells were seeded on 48-well plates (1.2×10^5 cells/well). After treatment with polyphenols and subsequent DQ-BSA, cells were scanned by Typhoon FLA9500 (GE Healthcare, Buckinghamshire, UK) at following settings: (λ ex: 473 nm, λ em: 520-540 nm). To correct differences in cell number during each treatment, cellular proteins were quantified using Protein Assay Bicinchoninic acid Kit (06385-00; Nacalai Tesque, Kyoto, Japan) to yield the ratio of fluorescence intensity/ cellular protein amounts.

For quantification of degradation products of DQ-BSA in conditioned medium, cells seeded on 12-well plates (6.0×10^6 cells/well) were treated with isorhamnetin (10

μM) or Torin1 (1 μM) for 24 h, then incubated with DQ-BSA (final 10 μg/mL) for 4 h. 50 μL of conditioned media was added into 450 μL of ice-cold methanol, then incubated at -20°C for 30 min for protein precipitation. After centrifugation (20,600 g, 4°C, 15 min), 10 μL of the supernatant was injected into a high-performance liquid chromatography (HPLC) column (0017201; TSKgel ODS-80Ts, 4.6 mm×150 mm, Tosoh Bioscience, Tokyo, Japan). The solvent system consisted of 0.1% trifluoroacetic acid (TFA) in 30% acetonitrile and 70% H_2O, and flow rate was 0.8 mL/min. Fluorescence detection was performed at following settings (λ ex: 505 nm, λ em: 515 nm). To prepare *in vitro* degradation products of DQ-BSA, 10 μg of DQ-BSA was incubated with 30 μg of pronase in 100 μL of 50 mM Tris-HCl buffer at 37°C for overnight. The sample was added into 800 μL of ice-cold methanol, and incubated at -20°C for 30 min. After centrifugation (20,600 g, 4°C, 15 min), 10 μL of the supernatant was injected into an HPLC column and analyzed in the way described above.

Fluorostaining of intracellular acidic compartment using Lysotracker

Cells were seeded into black-wall clear-bottom 96-well plates. After treatment with isorhamnetin (10 μM) or Torin1, mTOR inhibitor, (1 μM) for 24 h, cells were incubated with Lysotracker® Green DND-26 (L7526; Thermo Scientific, MA, USA) (final 0.5 μM) for 30 min. Following washing with FluoroBrite™ DMEM three times, green fluorescence from Lysotracker inside cells was imaged by using Operetta high-content imaging system at 40x magnification at following settings: (λ ex: 460-490 nm, λ em: 500-550 nm). Fluorescence intensity of each cell was quantified in the way described on DQ-BSA degradation assay.

Fluoroimaging of lysosomal cathepsin B activity

Magic Red® (937; Cosmo Bio, Tokyo, Japan) was dissolved into DMSO/water (1/1). Then, cells were seeded into black-wall clear-bottom 96-well plates. After treatment with isorhamnetin (10 μM) or Torin1 (1 μM) for 24 h, cells were incubated with Magic Red solution (2 μL/well) for 10 min. Following washing with FluoroBrite™ DMEM twice, cells were imaged by using Operetta high-content imaging system at 40x magnification at following settings: (λ ex: 530-560 nm, λ em: 570-650 nm). Red fluorescence intensity from digested Magic Red reagent inside each cell was quantified in the way described above.

Quantitative analyses of flavonols in cells

Cells were seeded on 12-well plates, and treated with each flavonol (10 μM) for 1 or 24 h. After washing cells with PBS five times, they were lysed with 300 μL of 1% acetic acid in methanol, containing flavone (10 nM) as an internal standard. Lysates were sonicated for 1 min and centrifuged at 20,600 g for 10 min for protein precipitation, and the supernatants were collected for liquid chromatography tandem mass spectrometry (LC-MS/MS) analyses. Quantitative analyses were performed using HPLC system interfaced to API3200 instrument (SCIEX, MA, USA) working with triple quadrupole analyser in multiple reaction monitoring (MRM) mode. 5 μL of each sample solution was injected into an HPLC column (CD024F; CD-C18 MF, 2 mm×100 mm, Imtakt, Kyoto, Japan). The solvent system consisted of 0.1% FA in H_2O (solvent A) and 0.1% FA in acetonitrile (solvent B). The gradient program was as follows: 0-2 min, 25% B; 2-10 min, linear gradient to 100% B; 10-15 min, 100% B hold; flow rate, 0.2 mL/min. The ESI-MS and MS/MS analysis were performed in negative mode. The ESI-MS source parameters

were optimized as follows: curtain gas, 10 psi; ion source gas 1, 30 psi; ion source gas 2, 80 psi; temperature, 700°C; and ion spray voltage, -4500 V, collision activated dissociation, 5 psi. The MS/MS transitions of *m/z* 314.932 → 300.000, 314.932 → 300.000, 284.845 → 92.900, 300.849 → 151.100, and 316.780 → 151.100 were for isorhamnetin, tamarixetin, kaempferol, quercetin and myricetin respectively.

Microscopic observation of intracellular GFP-tagged TFEB

Cells seeded on 12-well plates were transfected with pEGFP-N1-TFEB plasmid (38119; Addgene Inc., MA, USA) using Xfect™ Transfection Reagent. After recovery for 24 h, cells were reseeded into black-wall clear-bottom 96-well plates, and treated with isorhamnetin (10 μM) or Torin1 (1 μM) for 1 h. Hoechst 33342 was added into media (final 1 μg/mL) for staining nuclei, then cells were washed with FluoroBrite™ DMEM twice. Fluorescent image was captured by using Operetta high-content imaging system at 40x magnification at following settings: (GFP; λ ex: 460-490 nm, λ em: 500-550 nm, Hoechst 33342; λ ex: 355-385 nm, λ em: 430-500 nm).

Real-time reverse-transcription polymerase chain reaction

Total RNA, prepared using Sepasol®-RNA I Super G (09379-55; Nacalai Tesque, Kyoto, Japan), was reverse transcribed by ReverTra Ace® qPCR RT Master Mix with gDNA Remover (FSQ-301; Toyobo, Osaka, Japan). Then, cDNA was used as template for real-time quantitative polymerase chain reaction (RT-qPCR) analysis with THUNDERBIRD® SYBR qPCR Mix (QPS-201; Toyobo, Osaka, Japan) using LightCycler® Nano System (Roche Diagnostics K.K., Tokyo, Japan). The relative expression of each mRNA was calculated according to the $2^{-\Delta\Delta}$Ct method. GAPDH was

considered as internal control gene. The used primer sequences were as follows: ATPase H+ transporting V0 subunit d2 (Atp6v0d2), forward 5'- AAGCCTTTGTTTGACGCTGT -3' and reverse 5'- GCCAGCACATTCATCTGTACC -3'; cathepsin D (Ctsd), forward 5'- CTGAGTGGCTTCATGGGAAT -3' and reverse 5'- CCTGACAGTGGAGAAGGAGC -3'; glyceraldehyde-3-phosphate dehydrogenase (Gapdh), forward 5'- GTGAAGGTCGGAGTCAACG -3' and reverse 5'- TGAGGTCAATGAAGGGGTC -3'; lysosomal-associated membrane protein 2a (Lamp2a), forward 5'- GCAGTGCAGATGAAGACAAC -3' and reverse 5'- AGTATGATGGCGCTTGAGAC -3'.

Western blotting

Cells were washed with PBS, and lysed in a radio-immunoprecipitation assay buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.25% deoxycholic acid, 1% NP-40, 0.1% sodium dodecyl sulfate (SDS), 1 mM ethylenediaminetetraacetic acid] supplemented with both protease and phosphatase inhibitor cocktails. The protein concentrations in cell lysates were measured by BCA assay. Proteins were separated on a 10% SDS/polyacrylamide gel, then transferred to Immobilon-P PVDF membranes (IPVH00010; Millipore, MA, USA). The membrane was blocked with EzBlock Chemi (2332615; Atto Corp., Tokyo, Japan) at room temperature for 30 min, then probed with a primary antibody (1:1000) in Tris-buffered saline with 0.05% Tween 20 (TTBS) containing 5% BSA at 4 °C overnight. After washing with TTBS, they were incubated in solution containing secondary antibody conjugated with horseradish peroxidase (1:2000) at room temperature for 75 min. After washing, chemiluminescent detection was performed using Chemi-Lumi One or Chemi-Lumi One Super (07880, 02230; Nacalai

Tesque, Kyoto, Japan) and visualized by Ex-Capture MG (Atto Corp., Tokyo, Japan).

Statistical analysis

All data were expressed as means ± SEM. Statistical analysis were performed using *t*-test or one-way analysis of variance (ANOVA). When ANOVA indicated a significant difference among groups, these groups were compared by Dunnett's or Tukey's test. All statistical analyses were performed using Prism 5 (GraphPad Software, CA). The threshold for statistical significance was set at $p < 0.05$.

Results

Screening of dietary polyphenols which enhance lysosomal proteolysis in J774.1 cells

First, we attempted to establish a cell-based system to measure the activity of lysosomal proteolysis. We used DQ-BSA, BSA protein chemically modified with a kind of boron dipyrromethene (BODIPY) dye, for the assay system. This reagent is designed to be internalized into the cytoplasm to be degraded in lysosomes eventually, which is accompanied by the robust increase of fluorescence emission. DQ-BSA treatment to J774.1 mouse macrophage-like cells immediately increased the intracellular green fluorescence [Fig. 1a], suggesting its proteolytic degradation. To confirm whether the fluorescence intensity is indeed available as a quantitative indicator of lysosomal activity, we quantified the cellular fluorescence intensity after pre-treatment with Torin1, an mTOR inhibitor, which had been reported to facilitate DQ-BSA degradation in several cultured cell lines, and bafilomycin A1 (BAF), a chemical inhibitor of lysosomal acidification [18, 19] [Fig. 1a and b]. Expectedly, inhibition of lysosomal activity by BAF treatment resulted in the remarkable reduction of DQ-BSA-derived fluorescence, whereas

Torin1 significantly increased the intensity similarly to the previous reports.

Then, 56 kinds of dietary polyphenols, including flavonol, catechin, flavanone, anthocyanidin, flavanol, flavone, stilbene, isoflavone and chalcone, were treated to J774.1 cells respectively for the assessment of their effects on the lysosomal proteolysis (n=1). This screening test was performed at 10 μM concentration for all compounds because few of them showed cytotoxicity at up to this concentration upon 24 h treatment [data not shown]. We found that the treatment of a few flavonol compounds, including isorhamnetin, tended to enhance DQ-BSA degradation [Fig. 1c]. To verify the inducing activity of isorhamnetin on this assay system, we further performed DQ-BSA assay under several different experimental conditions, which revealed that treatment of isorhamnetin at relatively high concentrations (10 or 20 μM) significantly enhanced DQ-BSA degradation [Fig. 1d]. Additionally, it was found that its treatment for a shorter time (1-12 h) was not fully effective to enhance DQ-BSA digestion [Fig. 1e]. These results demonstrated that 24 h treatment of isorhamnetin, a kind of dietary flavonols, promote lysosomal proteolysis in J774.1.

Isorhamnetin activated lysosomal functions in J774.1 cells.

Given the possibility that isorhamnetin may enhance DQ-BSA intake through endocytosis as well as its lysosomal proteolysis, we attempted to assess the lysosomal properties by using other methodologies. Staining cells with lysotracker revealed that the area of acidic compartments per cell were significantly increased by isorhamnetin treatment [Fig. 2a and b], suggesting increased number of lysosomes. Regarding the proteolytic activity of lysosomes, the enzymatic activity of cathepsin B, one of lysosomal cysteine proteases, was evaluated by using a fluorescent substrate, Magic Red, showing

its significant activation by isorhamnetin treatment [Fig. 2c and d].

Interestingly, we also found that conditioned media of J774.1 cells treated with DQ-BSA emitted the remarkable green fluorescence, implying that the degradation products of DQ-BSA might be partly secreted into the media. To validate this hypothesis, we established another analytical methodology using HPLC-Fluorescence system which is capable to detect the final proteolytic products of DQ-BSA specifically. Proteolytic product of DQ-BSA, yielded by the *in vitro* incubation with pronase, was successfully detected as a single peak by the HPLC-Fluorescence analysis [Fig. 2e]. Expectedly, conditioned media of cells treated with DQ-BSA was found to contained fluorescent molecules with an almost identical retention time [Fig. 2f], and it was also notable that isorhamnetin treatment increased its extracellular secretion [Fig. 2g]. Furthermore, we confirmed that the enhancement of DQ-BSA degradation by isorhamnetin was observed in other mouse macrophage-like cell line, RAW264.7 [Fig. 2h]. These results confirmed that isorhamnetin treatment on murine macrophage-like cells led to the lysosomal activation.

3'-Methoxy group in B-ring moiety is critical for isorhamnetin activity

Based on the screening test shown in Figure 1c, isorhamnetin, a flavonol compound possessing mono-methoxylated B-ring, seemed to be the most effective among 56 candidates. Focusing on its characteristic structure of B-ring, the activities of 4 other flavonols with slightly differed B-ring (tamarixetin, kaempferol, quercetin and myricetin; Fig. 3a) were re-evaluated in triplicate by DQ-BSA assay. Intriguingly, these flavonol compounds, whose B-ring moieties were structurally varied, were shown to be inactive regarding to lysosomal proteolysis [Fig. 3b]. This result indicated the biological

significance of the chemical structure, 3'-methoxy-4'-hydroxy groups, on B-ring moiety in isorhamnetin.

Since the molecular polarities and stabilities of these 5 flavonols were speculated to be slightly differed, we attempted to quantify the amount of each compound accumulated in the cells after 1 or 24 h treatment. LC-MS/MS analysis of cell lysates revealed that 4 kinds of flavonols other than myricetin were interacted with cells after 1 h treatment. After 24 h treatment, quercetin was markedly reduced, whereas compounds with relatively hydrophobic B ring moiety, isorhamnetin, tamarixetin, and kaempferol, kept interacted with cells. [Fig. 3c and d]. It was noted that tamarixetin and kaempferol seemed to possess higher affinities for cells in spite of their less activities. It was also interesting that small amounts of isorhamnetin (0.07±0.006 nmol/mg protein; 1 h, 0.01±0.003 nmol/mg protein; 24 h) were detected in cells treated with quercetin, suggesting the *O*-methylation by catechol *O*-methyl transferase [20]. On the other hand, glucuronide or sulfate conjugates of these flavonols were not detected by HPLC-UV analyses (data not shown), indicating that these polyphenolic compounds are scarcely metabolized by conjugation reactions in this macrophage-like cell line, which has been reported to express β-glucuronidase to deconjugate flavonoid glucuronides [21].

These data suggest that a certain degree of hydrophobicity of B ring moiety is required to interact with cells, and 3'-methoxylated structure is critical for inducing lysosomal activation.

Isorhamnetin did not induce TFEB transactivation.

We next attempted to investigate the molecular mechanism underlying the inducing effects of isorhamnetin on lysosomal activity. The time-course experiment

shown in Figure 1e had revealed that 24 h treatment with isorhamnetin was indispensable for exhibiting its activity, which strongly suggested the modulation of gene expressions might be involved in the mechanisms. Consistent with this hypothesis, co-treatment of actinomycin D, an inhibitor of RNA synthesis, completely canceled the lysosomal activation induced by isorhamnetin treatment [Fig. 4a]. Taken together, altering gene expressions is considered to be essential for the lysosomal activation by isorhamnetin.

Considering the upstream mechanism of mRNA upregulation by isorhamnetin, we focused on TFEB, a transcriptional factor known as a master regulator of lysosomal biogenesis, whose activation has been reported to be controlled by mTORC1 signaling. To examine TFEB transactivation, we first observed its cellular localization in J774.1 cells. Fluoroimaging cells expressing TFEB tagged by GFP revealed its diffused localization in cytoplasm. Unexpectedly, isorhamnetin treatment scarcely promote the nuclear translocation of GFP-TFEB while its nuclear translocation was obviously seen in Torin1-treated cells [Fig. 4b]. On the other hand, lysosomal gene expressions such as *Atp6vod2*, *Ctsd* and *Lamp2a* were confirmed to be upregulated by overexpression of a constitutively active mutant form of TFEB (TFEB-S3A/R4A) [Fig. 4c]. Importantly, mRNA expressions of these TFEB target genes were not induced by isorhamnetin treatment whereas Torin1 treatment significantly increased their expressions [Fig. 4d]. Furthermore, isorhamnetin scarcely altered phosphorylation level of 4E-BP1, an mTORC1 substrate, in contrast to Torin1 [Fig. 4e]. These data showed mTORC1-TFEB signaling is not modulated by isorhamnetin, and suggested the possibility that lysosomal functions in J774.1 macrophages could be regulated partly through the distinct mechanism from TFEB-related pathway.

Discussion

Lysosome plays the pivotal role in the ultimate degradation on the catabolic processes through the endocytic and autophagic pathways. Whereas the effects of several dietary polyphenols on autophagy have been recently studied [12, 13, 14], there has been few reports paying attention to their effects on the lysosomal function. In this study, we performed the screening test on 56 kinds of compounds to find out dietary polyphenols which enhance the lysosomal proteolysis and identified isorhamnetin as an active compound.

Regarding the bioactivities of natural polyphenolic compounds, anti-oxidant and anti-inflammatory effects have been widely studied by both *in vitro* & *in vivo* experiments. Most of previous reports have shown that the anti-oxidant and anti-inflammatory activity of isorhamnetin was lower than that of quercetin, a 3'-*O*-demethylated derivative, due to lack of catechol structure [22, 23]. Interestingly, our present study described that the effect of enhancing lysosomal activity was seen in only isorhamnetin rather than quercetin. This result strongly suggests that the molecular mechanism underlying the lysosomal activation by isorhamnetin is independent on its anti-oxidant and anti-inflammatory activities. Moreover, our study also indicated that the chemical structure of 3'-methoxy-4'-hydroxy groups on B-ring moiety is critical for isorhamnetin activity. Considering these results, the lysosomal activation by isorhamnetin may require its physiological interaction to a specific intracellular molecule; that is to say, a certain key molecule may be interacted with isorhamnetin specifically and acting as its functional target in this cultured cell model.

Isorhamnetin is a bioactive compound derived from herbal medicinal plants or vegetables such as potherb mustards, radish leaves and apples (without peels) [24]. It has

been reported to possess multiple bioactivities like suppressive effects on arteriosclerosis in Apo-E knockout mice fed a high fat diet and adipocyte differentiation induced by rosiglitazone, a peroxisome proliferator-activated receptor γ (PPARγ) agonist, in 3T3-L1 cells [25, 26]. Considering its low availability from dietary intake, it seems to be difficult to orally take sufficient amount of isorhamnetin, since its content in vegetables was shown to be much lower than quercetin, which is widely contained in vegetables. For instance, the total content of isorhamnetin glycosides in onion were reported to be approximately 8-22 mg/100 g DW whereas that of quercetin glycosides were 171-294 mg/100 g DW [27]. However, it is worth noting that isorhamnetin has also been identified as a metabolite of quercetin in mammalian serum, which is transformed through the methylation reaction at 3'-hydoroxy group on B-ring by catechol-*O*-methyl-transferase mainly in the liver tissue. Indeed, serum concentration of isorhamnetin in mice has been reported to reach to approximately 2 μM after 6 weeks of feeding a diet supplemented with quercetin (2 mg/g) [28]. These reports suggest the possibility of quercetin as a potent and practical activator of lysosome in *in vivo* models.

This study investigated the molecular mechanisms underlying the lysosomal activation in isorhamnetin-treated J774.1 cells. Although data demonstrated that *de novo* gene expressions are closely involved in the mechanism, isorhamnetin treatment failed to promote TFEB nuclear translocation and the subsequent upregulation of lysosomal gene expressions such as *Atp6v0d2, Ctsd* and *Lamp2a*. Consistent with these results, isorhamnetin treatment also failed to repress the kinase activity of mTORC1, a negative regulator of TFEB transactivation. These data suggested that TFEB is not responsible for the mechanism of lysosomal activation by isorhamnetin in this cell line. Importantly, TFEB belongs to the MITF/TFE family of transcription factors that includes

microphthalmia-associated transcription factor (MITF), transcription factor binding to IGHM enhancer 3 (TFE3) and transcription factor EC (TFEC), and these 4 transcription factors have been reported to share the role in regulating similar series of lysosomal genes [29, 30, 31]. However, it is predictable that 3 transcription factors other than TFEB are not participating in the mechanism of isorhamnetin, since the transactivation of these MITF/TFE families are found to be commonly controlled by mTORC1 signaling, similarly to that of TFEB.

A previous report has described that treatment with genistein, one of the dietary isoflavone, upregulated lysosomal related gene expressions such as cathepsin F and mucolipin 1, and promoted lysosomal biogenesis through TFEB transactivation in HeLa cells [17]. Although genistein has also been tested in our screening assay [Fig. 1c], it didn't facilitate the lysosomal degradation of DQ-BSA in J774.1 cells. The higher concentration (50 μM) of genistein, compared with the previous report, also failed to activate lysosomal function in our assay [data not shown]. These observations strongly supported our prediction that unknown mechanisms independent on TFEB or MITF/TFE family are critical for the lysosomal regulation in J774.1 macrophages.

In summary, our results indicate that isorhamnetin activates lysosomal function in J774.1, and the molecular mechanism is not related to mTORC1-TFEB signaling. It is likely that future studies on isorhamnetin will lead to the identification of the novel critical signaling pathway for the lysosomal regulation in macrophages.

Author contributions

K. O. and Y. T. designed and supervised this study. M. S. performed the experiments. M. M., H. O., H. Y-O., and T. H. assisted with the experiments and contributed to the

discussions. M. S. wrote the manuscript with the assistance of K. O. and Y. T..

Disclosure statement

No potential conflict of interest was reported by the authors.

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569

570 **Figure 1 Screening test revealed that isorhamnetin enhanced DQ-BSA degradation.**

571 J774.1 cells were treated with Torin1 (1 μM) for 24 h, then incubated with DQ-BSA (10 μg/mL) for 1
572 h with/without bafilomycin A1 (100 nM) (a and b). Cells were treated with 56 kinds of polyphenolic
573 compounds (10 μM each) for 24 h, then DQ-BSA was added to quantify the lysosomal proteolysis (c).
574 Cells were treated with isorhamnetin at indicated concentrations for 24 h prior to DQ-BSA exposure
575 (d). Cells were treated with isorhamnetin at 10 or 20 μM for different h as indicated prior to DQ-BSA

exposure (e). Green fluorescence inside cell was imaged and quantified using Operetta CLS™ and Harmony 4.5 (a-c) or Typhoon FLA9500 (d and e). Relative fluorescence intensities to DMSO control were graphed (b-e). Data represent means ± SEM of triplicate determinations (b, d and e). Different letters indicate significant differences by Tukey-Kramer test ($p<0.05$) (b). Statistical significance was analyzed using Dunnett's test (*$p<0.05$) (d and e). Abbreviations of polyphenolic compounds in figure 1c are as follows: 3G; 3-glucoside, Q; quercetin, EGCG; epigallocatechin gallate, ECG; epicatechin gallate, EGC; epigallocatechin, EC; epicatechin, 7G; 7-glucosidice, Me; methyl.

Figure 2　Isorhamnetin enhances the lysosomal number and proteolytic function.

J774.1 cells were treated with isorhamnetin (10 μM) or Torin1 (1 μM) for 24 h, then incubated with lysotracker (0.5 μM) for 30 min (a) or Magic Red® (2 μL/well) for 10 min (c). Fluorescence intensities inside cells were imaged and quantified by using Operetta CLS™ and Harmony 4.5 (b and d). Degradation products of DQ-BSA by pronase digestion were detected by HPLC-fluorescence analysis (e). Cells were treated with isorhamnetin (10 μM) or Torin1 (1 μM) for 24 h, then incubated with DQ-BSA (10 μg/mL) for 1 h. Conditioned media were analyzed by HPLC-fluorescence analysis, and digested DQ-BSA was quantified (f and g). RAW264.7 cells were treated with isorhamnetin (10 μM) or Torin1 (1 μM) for 24 h, then incubated with DQ-BSA (10 μg/mL) for 1 h. The fluorescence was quantified in the same way as Figure 1 (a) and (b), and relative fluorescence intensities to DMSO control were graphed (h). Data represent means ± SEM of triplicate determinations. Statistical significance was analyzed using the Dunnett's test (*$p<0.05$) (b, d, g and h).

Figure 3　Structure-activity relationship of isorhamnetin for the lysosomal activation.

Chemical structures of isorhamnetin and other 4 flavonols with varied B-ring moieties are shown (a). Cells were treated with indicated flavonols (10 μM each) for 24 h, then incubated with DQ-BSA (10

μg/mL) for 1 h. Intensities of cellular green fluorescence were quantified by Typhoon FLA9500 (b). Cells were treated with each flavonol (10 μM) for 1 or 24 h, and their amounts accumulated in cells were quantified by LC-MS/MS analysis (c). The relative amounts of flavonols after 24 h treatment to 1 h treatment were shown (d). Data represent means ± SEM of triplicate determinations (b-d). Different letters indicate significant differences by Tukey-Kramer test ($p<0.05$) (b and d). Statistical significance was assessed using unpaired *t*-test (*$p<0.05$) (c).

Figure 4　mTORC1-TFEB signaling is not activated by isorhamnetin treatment.

Cells were treated with isorhamnetin (10 μM) with/without actinomycin D (5 μM) for 24 h. After DQ-BSA exposure, cellular fluorescence intensities were quantified by Typhoon FLA9500 (a). GFP-TFEB expressing cells were treated with isorhamnetin (10 μM) or Torin1 (1 μM) for 1 h, then nuclei were stained with Hoechst33342. Cellular fluorescence was imaged by using Operetta CLS™ (b). Relative mRNA expressions of indicated lysosomal genes in cells overexpressing constitutively activated TFEB (TFEB-S3A/R4A) (c). Relative mRNA expressions of TFEB target genes after the incubation with isorhamnetin (10 μM) for 8 h (d). Phosphorylated forms of 4E-BP1 in cells treated with isorhamnetin (10 μM) or Torin1 (1 μM) for 1 h (e). Data represent means ± SEM of triplicate determinations (a, c and d). Different letters indicate significant differences by Tukey-Kramer test ($p<0.05$) (a). Statistical significance was analyzed using Dunnett's test (*$p<0.05$)

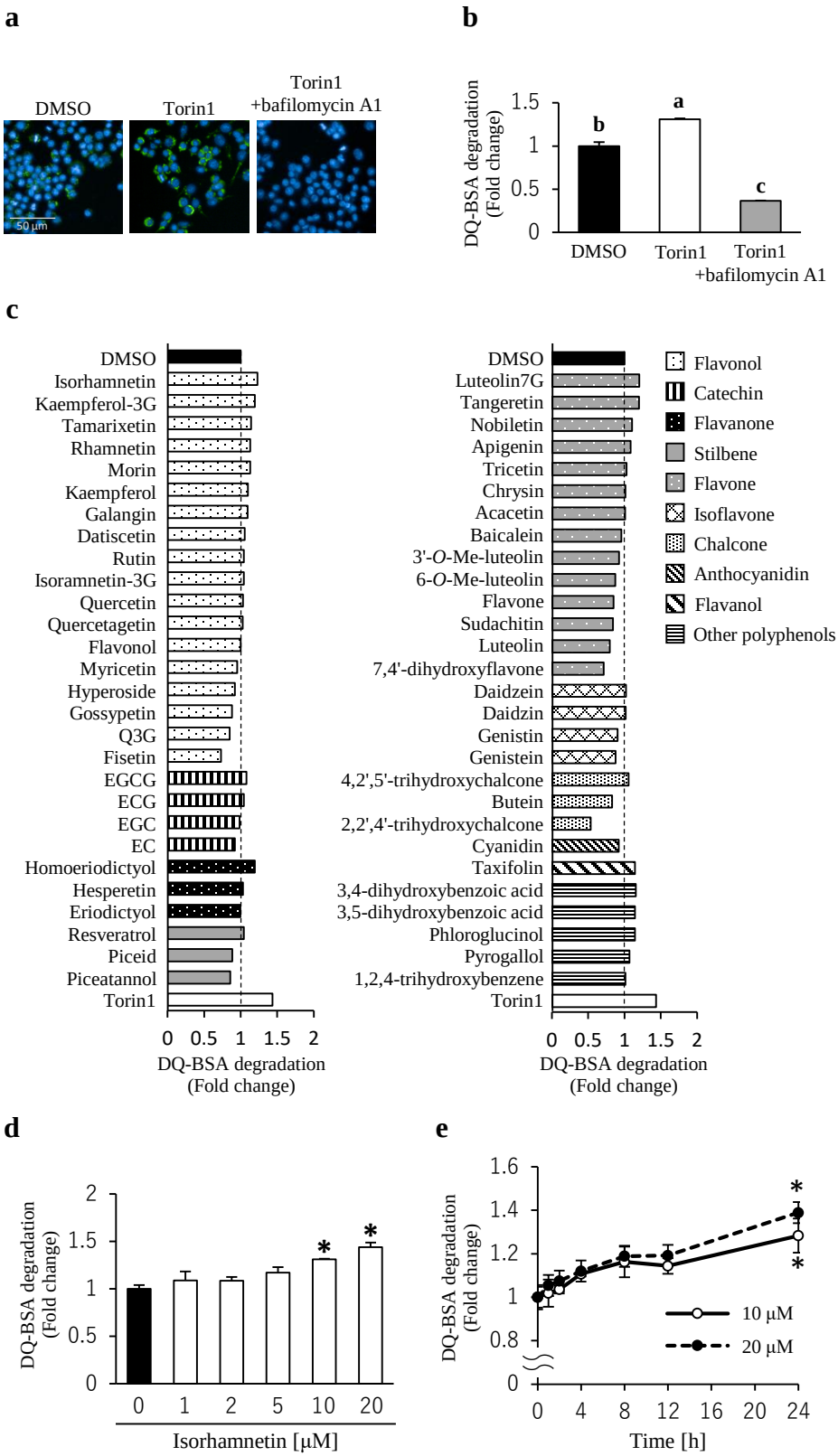


Figure 1, Sakai M *et al.*

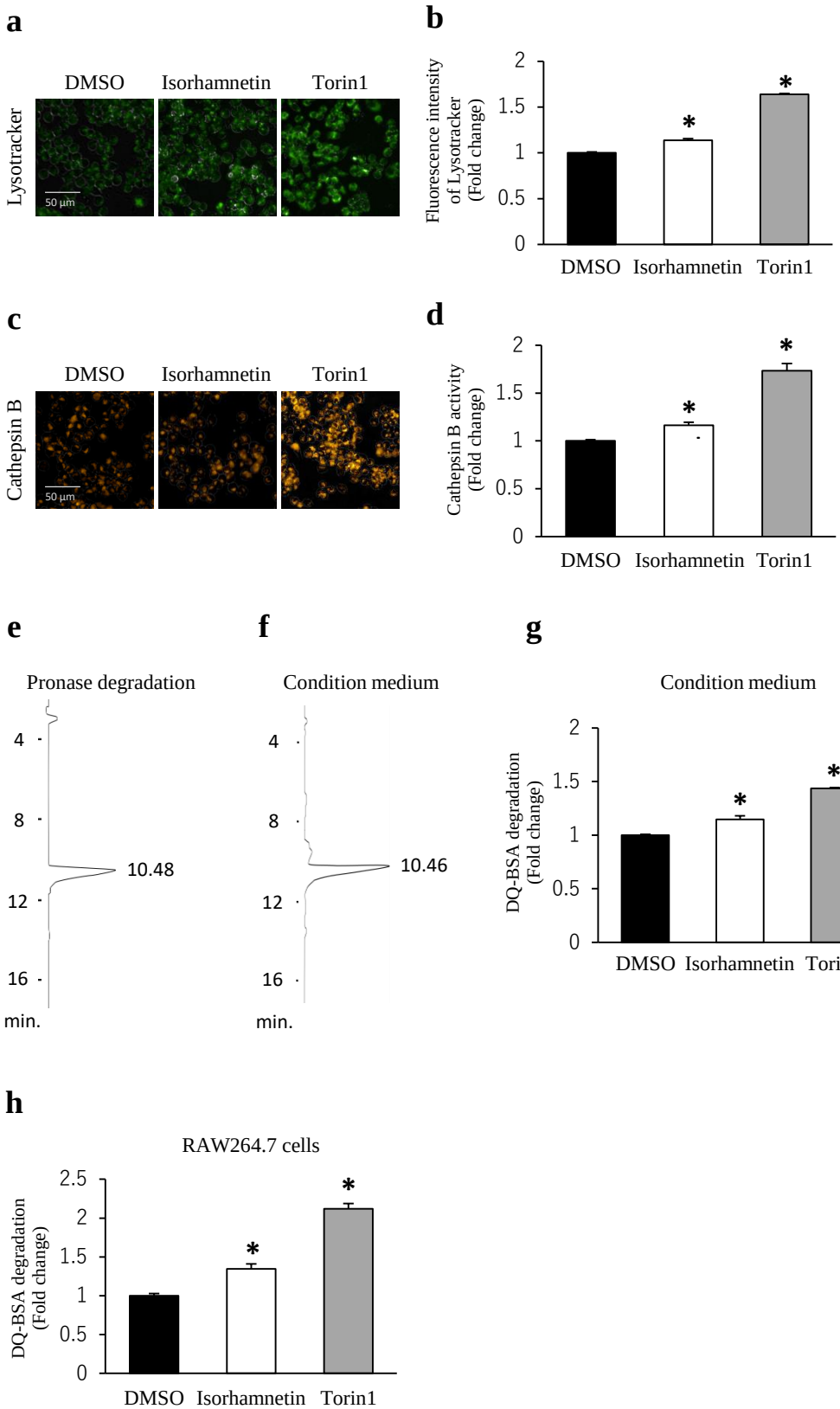
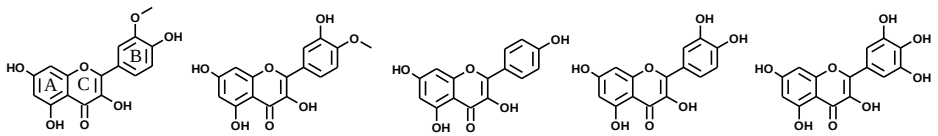


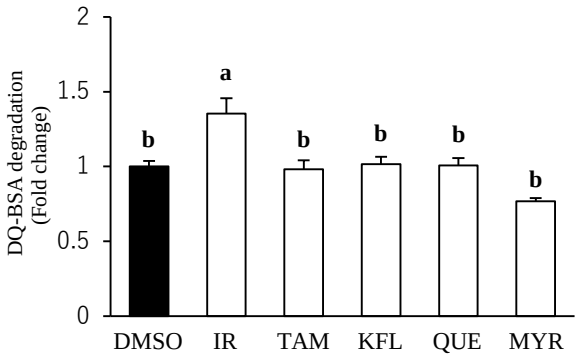
Figure 2, Sakai M *et al.*

a

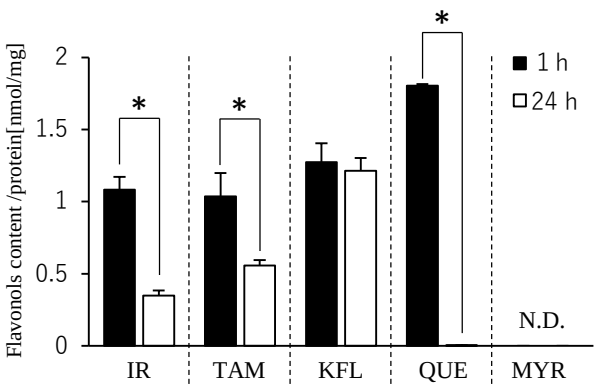


Isorhamnetin (IR) | Tamarixetin (TAM) | Kaempferol (KFL) | Quercetin (QUE) | Myricetin (MYR)

b



c



d

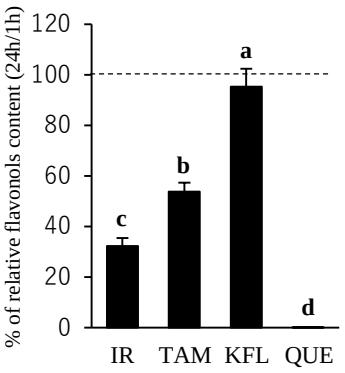


Figure 3, Sakai M *et al.*

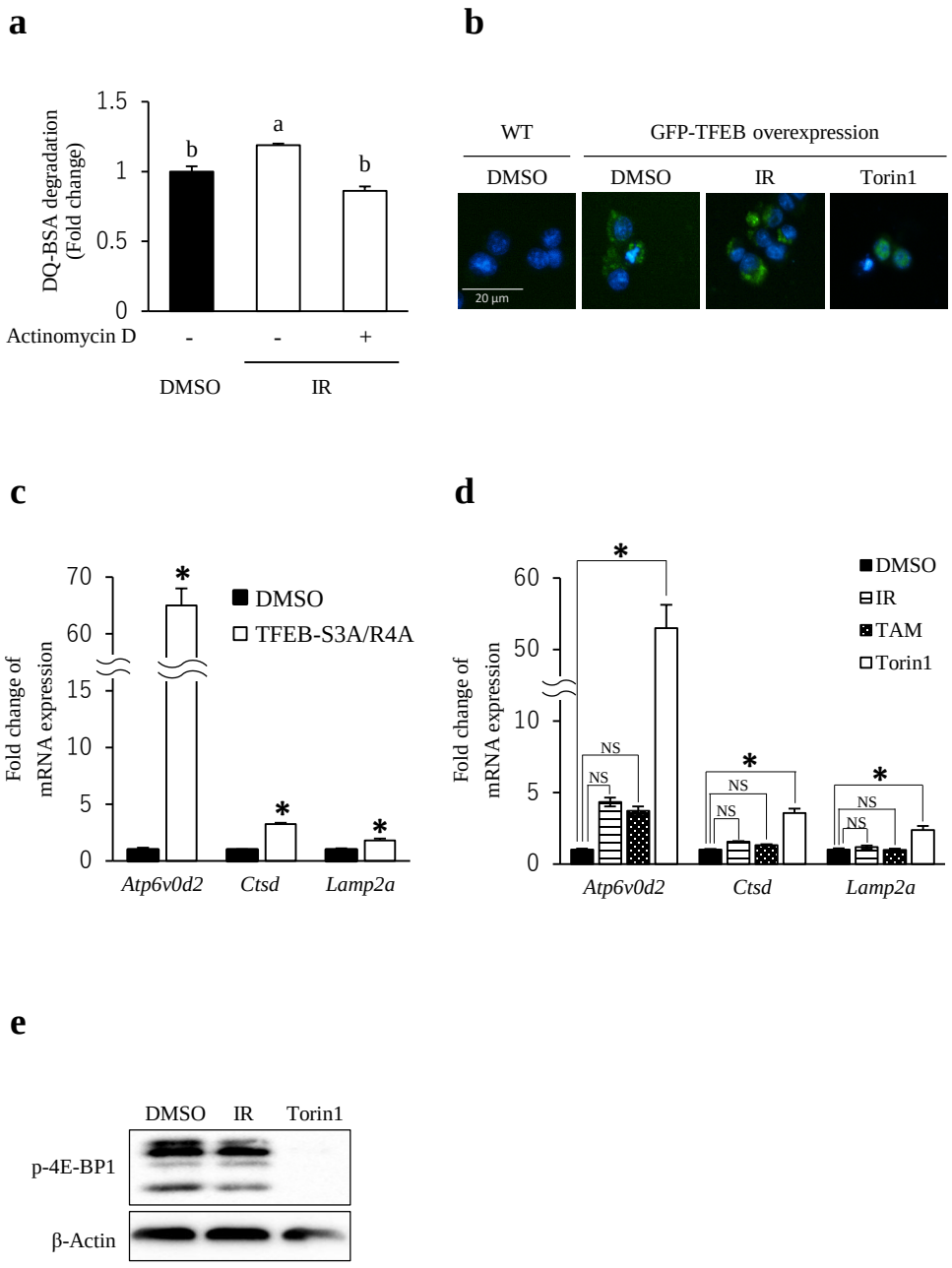


Figure 4, Sakai M *et al.*