Beneficial Effects of Xanthohumol on Obesity and Insulin Resistance in Obese, Diabetic (ob/ob) Mice

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Abstract

Xanthohumol (XN), the principal flavonoid from the hop plant, has anti-obesity on and anti-diabetic effects in rodent models of obesity and diabetes. However, the effects of XN on obesity and insulin resistance have not been fully characterized. Here, we show in obese, diabetic (ob/ob) mice that XN treatment (10 mg/kg BW, IP) for six weeks significantly decreased body weight and plasma insulin levels and improved glucose tolerance, although food intake and blood glucose levels were not altered. XN ameliorated fatty liver and significantly increased insulin-stimulated phosphorylation of Akt in the liver. Moreover, XN significantly decreased respiratory quotient (RQ), indicating increased fat oxidation by XN. These results indicate that the beneficial effects of XN were associated with improvements in fatty liver, hepatic insulin signaling and increased fat oxidation.

Introduction

Obesity is a pandemic in the United States and worldwide. Obesity is a major causative factor for type 2 diabetes. Type 2 diabetes is associated with hypertriglyceridemia, hypercholesterolemia and fatty liver and increases the risk of cardiovascular disease. Thus, obesity and type 2 diabetes have been a major public health issue.

Xanthohumol (XN) is the principal prenylated flavonoid from hops (Humulus lupulus) and a constituent of beer [1]. Previous studies have shown that XN has protective effects against obesity and diabetes. Treatment with XN decreased blood glucose levels in diabetic KK-Ay mice [2], high-fat diet (HFD)-fed mice [3] and obese, diabetic Zucker fa/fa rats [4], and decreased body weight gain in HFD-fed mice [3] and fa/fa rats [4]. A recent study has shown that hops extract reduces body weight in healthy overweight individuals [5]. However, the beneficial effects of XN on glucose and lipid metabolism have not yet been fully characterized. XN treatment significantly decreased plasma levels of insulin, total cholesterol and triglycerides in HFD-fed mice [3]. In contrast, XN did not alter these parameters in fa/fa rats [4], although XN decreased blood glucose level and body weight in fa/fa rats.

Similarly, the molecular mechanisms underlying the anti-obesity and anti-diabetic effects of XN are not fully understood. Previous studies have proposed different mechanisms. It has been reported that XN acts on farnesoid X receptor (FXR) [2] and increases mitochondrial uncoupling and glutathione recycling [6], contributing to the beneficial effects of XN on lipid metabolism and obesity. In addition,
XN decreases the mRNA expression of sterol regulatory element-binding protein (SREBP)-1 [7, 8], one of the master regulator of fatty acid and lipid production, and functions as an inactivator of SREBP-1 [9].

Activation of Akt plays a central role in the metabolic actions of insulin. We and others have shown that inducible nitric oxide synthases (iNOS) plays an important role in obesity-induced insulin resistance [10-12]. iNOS-mediated S-nitrosylation of Akt inactivates Akt [13]. Moreover, we have previously shown that hepatocyte-specific increased iNOS expression causes hepatic insulin resistance and fatty liver along with S-nitrosylation of Akt in mice [14]. However, the effects of XN treatment on insulin signaling or iNOS have not yet been studied. We, therefore, examined the effects of XN treatment on iNOS expression and S-nitrosylation of Akt in relation to hepatic insulin response and fatty liver.

Here, we studied various aspects of the insulin-sensitizing effects of XN in leptin-deficient obese, diabetic (ob/ob) mice.

Materials and Methods

Materials

Xanthohumol (XN) (Tokyo Chemical Industry, Tokyo, Japan), dimethyl sulfoxide (DMSO) (Wako Pure Chemical Industries, Osaka, Japan), methyl methanethiosulfonate (MMTS), ascorbate sodium, dithiothreitol (DTT), N-(6-(biotinamido)hexyl)-3’-(2’-pyridyldithio)-propionamide (HPDP-biotin), NeutrAvidin agarose resins (Pierce, Rockford, IL), and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Cell Signaling, Beverly, MA, USA), anti-uncoupling protein-1 (UCP1) (Abcam, Cambridge, UK), anti-Akt, anti-phospho-Akt (T308), anti-phospho-Akt (S473) (Cell Signaling) were purchased commercially.

Animals and animal care

Male ob/ob mice at eight weeks of age, purchased from the Sankyo Laboratory (Tokyo, Japan), were used for this study. We strictly followed the guidelines for the care and use of laboratory animals of Tokyo Medical and Dental University. The study was approved by the Institutional Animal Care Committee of Tokyo Medical and Dental University (No. 2013-092C, No. 0170190A). The animal care facility is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. The mice were maintained at 25°C and illuminated by 12-h light-dark cycles. The mice were provided free access to standard rodent chow and water ad libitum. The mice were treated with XN (10 mg/kg BW, IP) in the experimental group or DMSO (vehicle) in the control group 5 days/week from Monday to Friday for 6 weeks. Liver and epididymal adipose tissues were collected at 14 weeks of age under anesthesia with ketamine (100 mg/kg BW, IP) with xylazine (10 mg/kg BW, IP).

Glucose tolerance test

Glucose (1.0 g/kg BW) was intraperitoneally administered to ob/ob mice following overnight fasting at 13 weeks of age. Blood samples were collected just before and at 15, 30, 60 and 120 minutes after the glucose injection. Blood glucose levels were measured using the Glutest Neo Super device (Sanwa Kagaku Kenkyusho, Aichi, Japan). The plasma insulin concentrations were determined using an AKRIN-011S mouse insulin ELISA kit (Shibayagi, Gunma, Japan).

Assessment of insulin sensitivity according to the homeostasis model assessment (HOMA) and insulin resistance (IR) index

In order to assess the whole-body insulin sensitivity in ob/ob mice, the HOMA-IR index was determined using the HOMA2 Calculator software program (downloaded from www.OCDEM.ox.ac.uk) based on the blood glucose and plasma insulin concentrations at 14 weeks of age.

Measurement of resting energy expenditure

The whole-body energy expenditures of XN and DMSO treated groups were measured using a mass spectrometry system (ARCO 2000, Arco system, Chiba, Japan). Briefly, mice were individually put into a metabolic chamber after injection of xanthohumol or saline (IP), and monitored during dark period with ad libitum access to chow and water. Gas samples were collected and analysed every 3 minutes per animal. Output parameters include VO₂ (ml/min and ml/min/kg) and RQ (VO₂/VCO₂), and the data for 4 h during dark period were averaged.

Biotin-switch assay to detect the S-nitrosylation of Akt
The degree of S-nitrosylation of Akt was evaluated according to the biotin-switch assay, as previously described [14] with minor modifications. Briefly, the liver tissues were rinsed with phosphate-buffered saline (PBS), powdered under liquid nitrogen and homogenized in homogenization buffer (PBS-HCl, pH 3.5, 150 mM NaCl, 1 mM EDTA, 1 mM diethylenetriaminepentaacetic acid [DTPA], 2.5% SDS, 0.5% NP-40, 0.1 mM neocuproine, 80 M of carmustine, 1 mM PMSF, protease inhibitor cocktail [Sigma]). The homogenates were subsequently incubated at 50°C for 20 minutes with vortexing every two minutes following the addition of 1 volume of blocking buffer (PBS-HCl, pH 3.5, 150 mM NaCl, 1 mM EDTA, 1 mM DTPA, 2.5% SDS, 0.1 mM neocuproine, 40 mM MMTS). The proteins were precipitated with pre-chilled acetonitrile, dissolved in modified HENS buffer (25 mM HEPES, pH 7.7, 1% SDS, 1 mM EDTA, 1 mM DTPA, 0.1 mM neocuproine) and neutralized with HEN buffer (25 mM HEPES, pH 7.7, 0.5% Triton X-100, 1 mM EDTA, 1 mM DTPA, 100 mM NaCl, 0.1 mM neocuproine). The samples were then incubated with 4 mM HPDP-biotin in the presence of 4 mM ascorbate sodium for one hour at room temperature. After excess HPDP-biotin was removed via acetone precipitation, the samples were incubated with streptavidin-agarose beads for one hour at room temperature. The beads were then washed three times with wash buffer (25 mM HEPES, pH 7.7, 1 mM EDTA, 500 mM NaCl, 0.5% Nonidet P-40), and the biotinylated proteins were eluted via incubation with elution buffer (20 mM HEPES, pH 7.7, 1 mM EDTA, 100 mM NaCl, 200 mM DTT) for 30 minutes and separated via SDS-PAGE for immunoblotting with the anti-Akt antibody.

Evaluation of the effects of xanthohumol on hepatic insulin signaling

To assess the hepatic insulin sensitivity in ob/ob mice, we injected insulin via the portal vein in both XN and DMSO (vehicle) treated mice. At 8 weeks of age, the ob/ob mice were randomly assigned to XN and DMSO (vehicle) treatment groups. After 6-week the mice were fasted overnight at 14 weeks of age. Under anesthesia with pentobarbital (50 mg/kg BW, IP), insulin (0.5 units/Kg BW, Humulin R; Eli Lilly, Indiana, IN) or saline was injected via the portal vein. Five minutes after the injection, the liver was removed under anesthesia with ketamine (100 mg/kg BW, IP) with xylazine (10 mg/kg BW, IP) and snap frozen in liquid nitrogen.

Isolation of total RNA and quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated with an RNeasy Mini kit (Qiagen, Valencia, CA, USA). First-strand cDNA was synthesized from 1 μg of total RNA using a High-capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA, USA). Real-time RT-PCR analyses were performed as previously described [15] using 10 ng cDNA and TaqMan probes (Applied Biosystems) for sterol regulatory element-binding protein (Srebp)-1, acetyl CoA carboxylase (Acc), Fatty acid synthase (Fas), inducible nitric oxide synthase (iNos) and glyceraldehyde-3-phosphate dehydrogenase (Gapdh) using a TaKaRa PCR Thermal Cycler Dice (Takara Bio, Osaka, Japan). The gene expressions were normalized to that of Gapdh.

Immunoblotting

Tissue samples were homogenized as previously described [15], with minor modifications. Briefly, the tissues were homogenized in ice-cold homogenization buffer A (50 mM HEPES, pH 8.0, 150 mM NaCl, 2 mM EDTA, 2.5% lithium dodecylsulfate, 2% CHAPS, 10% glycerol, 10 mM sodium fluoride, 2 mM sodium vanadate, 1 mM PMSF, 10 mM sodium pyrophosphate, 1 mM DTT, and protease inhibitor cocktail). Following incubation at 4°C for 30 minutes, the homogenized samples were centrifuged at 13,000 g for 10 minutes at 4°C. Immunoblotting was subsequently performed as previously described [15]. ECL select reagent (GE Healthcare Life Science, Pittsburgh, PA, USA) was then used to visualize the blots, and bands of interest were scanned using the LAS 1000 (Fujifilm, Tokyo, Japan) and quantified using the Multi Gauge software program (Fujifilm).

Histological analyses

Immunostaining with anti-UCP1 antibody were performed on formalin-fixed paraffin-embedded sections by a standard protocol. For immunohistochemistry, we placed the pieces on coated glass slides (Immuno Coated Slide; Muto Pure Chemicals Co., Ltd., Tokyo, Japan). The sections were deparaffinized in xylene and ethanol. The slides were then autoclaved in 10 mM citrate buffer (pH 6.0) at 121°C for 15 minutes for antigen retrieval. The specimens were washed using PBS buffer and treated with 3% hydrogen peroxide for 5 minutes to block endogenous peroxidase activity. The specimens were washed in PBS and incubated for 1 hour at room temperature with anti-UCP1 antibody or isotype control IgG (Abcam, Cambridge, UK). The specimens were then washed with PBS and incubated using DAKO polymer solutions (Envision; DAKO, Carpinteria, CA, USA) for 30 minutes. The peroxidase activity was detected using DAB for 3 minutes. Finally, the nuclei were counterstained with Mayer’s hematoxylin solution. Pictures were taken under a microscope (BX53, Olympus, Tokyo, Japan) with a connected digital camera (DS-Fi2-L3, Nikon, Tokyo, Japan).
Measurement of the GSH/GSSG ratio

The reduced glutathione (GSH) to oxidized glutathione (GSSG) ratio was measured using the Bioxytech GSH/GSSG Assay kit (Percipio Biosciences, Foster, CA, USA) according to the manufacturer’s instructions. Briefly, the liver tissues were homogenized in 15-fold volumes of ice-cold 5% metaphosphoric acid (MPA), and the homogenates were centrifuged at 10,000 g for 20 minutes at 4°C. For the GSH analysis, MPA extracts were diluted 60-fold in assay buffer (Na·PO₄ with EDTA). The final concentration in the samples was 1/488. For the GSSG analysis, M2VP was mixed with the MPA extracts to inhibit the oxidation of GSH to GSSG, and the samples were neutralized by the addition of 5 µl of TEA and subsequently diluted 4-fold in 5% MPA and 15-fold in assay buffer. The final concentration in the samples was 1/60. The changes in absorbance at 412 nm were recorded for 3 minutes, and the GSH/GSSG ratio was calculated based on the formula described in the manufacturer’s instructions.

Measurement of the liver TG content

Lipid extraction from the liver tissues was performed as previously described [16]. The triglyceride content in the lipid extracts was measured using the Triglyceride E-test Wako kit (Wako Pure Chemical Industries, Osaka, Japan) according to the manufacturer’s instructions.

Measurement of the plasma triglyceride, total cholesterol and free fatty acids concentrations

The plasma triglyceride, total cholesterol and free fatty acids concentrations were measured respectively using the Triglyceride E-test Wako kit (Wako), Cholesterol E-test Wako kit (Wako) and NEFA C-test Wako kit (Wako) according to the manufacturer’s instructions.

Statistical Analyses

The data were analyzed by a one-way analysis of variance followed by Scheffe’s multiple comparison test or Student’s t-test. A p value of < 0.05 was considered to be statistically significant. All values are expressed as the mean ± SEM.

Results

Treatment with xanthohumol (XN) decreased body weight gain and plasma insulin concentration in obese, diabetic (ob/ob) mice.

Treatment with XN for 6 weeks significantly decreased body weight in ob/ob mice compared with vehicle alone (Figure. 1(a)). However, XN treatment did not alter food intake (Figures. 1(b) and 1(c)). XN treatment did not decrease blood glucose level, but significantly decreased plasma insulin concentration after 4 hours fasting in ob/ob mice (Figs. 1(d) and 1(e)).
XN decreased body weight of ob/ob mice at 14 weeks of age compared with vehicle (a) without decreasing food intake (b, c). XN did not alter blood glucose level (d), but significantly decreased plasma insulin concentration (e) and HOMA-IR (f) after 4 hours fasting. Glucose (1.0 g/kg BW) was intraperitoneally administered to ob/ob mice following overnight fasting at 13 weeks of age. Glucose tolerance test (GTT) showed that blood glucose level at 120 minutes after glucose injection was significantly lower in XN-treated mice compared with vehicle (g). Area under the curve analysis also showed decreased blood glucose levels during GTT (h). n = 6-7 mice per group. *p < 0.05, **p < 0.01 vs. Vehicle.

The homeostatic model assessment of insulin resistance (HOMA-IR), an indicator of insulin sensitivity, was significantly decreased by XN treatment relative to vehicle alone (Figure. 1(f)). These results indicate that XN treatment ameliorated insulin resistance in ob/ob mice. Consistently, glucose tolerance test showed that XN treatment improved glucose tolerance in ob/ob mice (Figs. 1(g) and 1(h)). XN treatment tended to decrease blood glucose level, however, there were not significant difference between XN and vehicle treatment after over night fasting (p = 0.07).

XN treatment decreased triglycerides content in the liver of ob/ob mice.

XN treatment significantly decreased triglycerides content in the liver of ob/ob mice compared with vehicle alone (Figure 2(a)). However, XN treatment did not significantly decrease plasma triglycerides level in ob/ob mice (Figure 2(b)). On the other hand, XN treatment significantly decreased plasma total cholesterol levels (Figure 2(c)), while plasma free fatty acid concentration was not altered by XN treatment (Figure 2(d)).

Next, we examined the effects of XN treatment on expression of genes involved in fat synthesis in the liver. XN treatment did not alter Srebp-1 mRNA level (Figure 2(e)), but significantly decreased acetyl CoA carboxylase (Acc) mRNA level compared with vehicle alone (Figure 2(f)). Fatty acid synthase (Fas) mRNA level appeared to be decreased by XN treatment, but there was no statistically significant difference between the two groups (Fig. 2(g)).

![Figure 2. Effects of XN on parameters of lipid metabolism.](image)

XN significantly decreased triglycerides content in the liver compared with vehicle (a). XN significantly decreased plasma cholesterol level (c), while XN did not significantly decrease plasma triglycerides (b) and free fatty acid levels (d). XN significantly decreased Acc mRNA level in the liver (f), while Srebp-1 and Fas mRNA levels were not significantly altered by XN (e, g). n = 6-7 mice per group. *p < 0.05, **p < 0.01 vs. Vehicle. N.S.: not significant.
XN treatment increased insulin-stimulated Akt phosphorylation in the liver of ob/ob mice.

XN treatment significantly increased insulin-stimulated phosphorylation of Akt at threonine 308 and serine 473 compared with vehicle alone (Figure 3). In rodent models of obesity-induced diabetes [17-19] and hepatocyte-specific iNOS transgenic mice [14], basal (exogenous insulin-naïve) Akt phosphorylation is increased, which is in part related to hyperinsulinemia. We, therefore, studied the effects of XN on basal Akt phosphorylation as well. When saline, but not insulin, was injected, basal (exogenous insulin-naïve) phosphorylation of Akt appeared to be lower in XN-treated mice than vehicle-treated mice. However, there was no statistically significant difference in basal Akt phosphorylation between the two groups. Akt protein expression was not altered by XN (Figure 4(b)). These results indicate that XN treatment improved hepatic insulin signaling.

In association with increased insulin-stimulated Akt phosphorylation, XN treatment significantly decreased iNOS expression, S-nitrosylation of Akt in the liver of ob/ob mice (Figure 4).

![Figure 3. XN increased insulin-stimulated Akt phosphorylation in the liver.](image)

Insulin-stimulated Akt phosphorylation at threonine 308 (a, c) and at serine 473 (a, d) was significantly increased by XN compared with vehicle. Akt protein expression was not altered by XN (b). n = 6 mice per group. **p < 0.01, ***p < 0.001 vs. Saline. †p < 0.05, †††p < 0.001 vs. Vehicle+Insulin, N.S.: not significant.
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Figure 4. Effects of XN on iNOS expression and S-nitrosylation of Akt in the liver.

XN significantly decreased iNOS mRNA expression (a) and S-nitrosylated (SNO) Akt (b) in the liver compared with vehicle. n = 6-7 mice per group. *p < 0.05, **p < 0.01 vs. Vehicle.

Effects of XN on glutathione content in the liver.

A previous study indicated that XN alters glutathione metabolism [6]. XN treatment increased GSH content in the liver (Figure 5(a)). On the other hand, XN treatment did not significantly alter oxidized glutathione (GSSG) and GSH-to-GSSG ratio (Figs. 5(b) and 5(c)). XN treatment increased UCP1 expression in white adipose tissue and fat oxidation.

Figure 5. Effects of XN on glutathione levels in the liver.

XN significantly increased GSH compared with vehicle (a). GSSG and GSH-to-GSSG (GSH/GSSG) ratio were not significantly altered by XN (b, c). n = 6-7 mice per group. *p < 0.05 vs. Vehicle.

Increased uncoupling protein 1 (UCP1) contributes to inhibition of body weight gain and promotion of fat oxidation in white adipose tissue [20]. We, therefore, examined the effects of XN on UCP1 expression. XN treatment significantly increased UCP1 expression in white adipose tissue compared with vehicle alone (Figure 6).
Next, we evaluated the effects of XN on energy expenditure. XN treatment did not decrease total oxygen consumption (VO₂) (Figure 7(a)). When normalized to body weight, the mean of oxygen consumption (VO₂/body weight) was greater in XN-treated ob/ob mice compared with vehicle alone. However, there was no statistically significant difference between the two groups (Figure 7(b)). XN treatment significantly decreased respiratory quotient (RQ) compared with vehicle alone (Figure 7(c)). RQ is the ratio between VO₂ and VCO₂ and indicates energy substrate being oxidized. The decreased RQ in XN-treated mice indicates that XN treatment increased fat oxidation in ob/ob mice.

![Figure 6. Effects of XN on UCP1 expression in white adipose tissue.](image)

Immunoblotting (a) and immunohistochemistry (b) revealed that XN increased UCP1 expression in white adipose tissue compared with vehicle. n = 6-7 mice per group. ***p < 0.001 vs. Vehicle. The scale bars show 50 μm.

![Figure 7. Effects of XN on resting energy expenditure.](image)

XN did not significantly alter VO₂ and VO₂ normalized to body weight (VO₂/BW) (a, b). On the other hand, XN significantly decreased RQ compared with vehicle (c). n = 6-7 mice per group. *p < 0.05 vs. Vehicle.

**Discussion**

Our data show in ob/ob mice that XN treatment: (1) decreased body weight; (2) improved insulin sensitivity and glucose tolerance; (3) increased insulin-stimulated Akt phosphorylation; (4) decreased triglycerides content in the liver; (5) increased UCP1 expression in white adipose tissue; and (6) decreased RQ. These data are largely consistent with previous studies showing the anti-obesity and anti-diabetic effects of XN [2-4]. The salient new findings in this study are that XN treatment: (a) increased insulin-stimulated Akt phosphorylation; (b) decreased iNOS expression and S-nitrosylation of Akt in the liver; (c) increased UCP1 expression in white adipose tissue; and (d) increased fat oxidation as indicated by decreased RQ in ob/ob mice.

Our previous study has shown that hepatocyte-specific increased iNOS expression causes insulin resistance and fatty liver in mice [14].
Our data indicate that XN treatment improved hepatic insulin resistance, which paralleled decreases in iNOS expression and S-nitrosylation of Akt. Together, it is conceivable that mitigation of iNOS-mediated nitrosative stress contributes to the beneficial effects of XN in ob/ob mice.

The molecular mechanisms underlying the beneficial effects of XN are incompletely understood. A previous study suggested that mitochondrial uncoupling and increased glutathione recycling may be a contributor to the protective effects of XN [6]. Here, we provide a piece of evidence that supports this notion, namely increased UCP1 protein. It is possible that increased UCP1 expression in white adipose tissue might contribute to an increase in mitochondrial uncoupling. In addition, one can speculate that increased UCP1 expression in white adipose tissue may play a role in XN-induced increased fat oxidation, which, in turn, contributes to anti-obesity effects of XN in ob/ob mice. Moreover, increased GSH may be possibly related to increased glutathione recycling.

SREBP-1 is a transcription factor that plays a key role in the development of fatty liver [21]. It has been reported that XN suppresses mRNA expression of Srebp-1 in the liver of rodents [7, 8]. Inactivation of SREBP-1 has also been proposed as a mediator of the protective effects of XN [9]. Our results showed that XN did not alter the expression level of Srebp-1. However, XN treatment significantly decreased mRNA expression of Acc, a target gene of Srebp-1. It is not clear why XN did not suppress the mRNA expression of Srebp-1 in our study, however one of the possibilities is related to the dosage of XN treatment. In our study, we treated with XN at a dose of 10 mg/kg/day. On the other hand, in previous studies by others they treated with 100 to 300 mg/kg/day of XN [2, 7-9]. It is possible, therefore, the low dose treatment of XN (10 mg/kg/day) might decrease the transcriptional activity of SREBP-1 but not mRNA expression levels in this study. These data suggest that XN may attenuate the transcriptional activity of SREBP-1 in the liver, thereby leading to the amelioration of fatty liver in ob/ob mice. However, further studies are required to clarify the precise mechanisms of anti-obesity and insulin-sensitizing effects of XN.

In summary, XN treatment elicited beneficial effects in the liver and adipose tissue, including reductions in triglycerides content and Acc and iNos expression in the liver and increased UCP1 expression in white adipose tissue. It is conceivable that these effects in the liver and adipose tissue may contribute in concert to the anti-obesity and insulin-sensitizing effects of XN in ob/ob mice.

**Conclusion**

XN treatment significantly ameliorated insulin resistance and decreased body weight in obese, diabetic (ob/ob) mice. The insulin-sensitizing effects of XN were associated with improvements in fatty liver, and hepatic insulin signaling and increases in fat oxidation and UCP1 expression in white adipose tissue.

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