Mice Heterozygous for the Xanthine Oxidoreductase Gene Facilitate Lipid Accumulation in Adipocytes

Noboru Murakami, Toshio Ohtsubo, Yasuo Kansui, Kenichi Goto, Hideko Noguchi, Yoshie Haga, Yusaku Nakabeppu, Kiyoshi Matsumura, Takanari Kitazono

Objective—Xanthine oxidoreductase (XOR) catalyzes the production of uric acid with concomitant generation of reactive oxygen species. XOR has been shown to regulate adipogenesis through the control of peroxisome proliferator–activated receptor γ, but its role in adipose tissue remains unclear. The aim of this study was to examine the role of XOR in adipose tissue using XOR genetically modified mice.

Approach and Results—Experiments were performed using 2-, 4-, and 18-month-old XOR heterozygous mice (XOR+/−) and their wild-type littermates to evaluate the physiological role of XOR as the mice aged. Stromal vascular fraction cells were prepared from epididymal white adipose tissue in 2-month-old XOR mice to assess adipogenesis. At 18 months, XOR+/− mice had significantly higher body weight, higher systolic blood pressure, and higher incidence of insulin resistance compared with wild-type mice. At 4 months, blood glucose and the expressions of CCAAT enhancer–binding protein β, peroxisome proliferator–activated receptor γ, monocyte chemoattractant protein-1, and tumor necrosis factor α mRNA in epididymal white adipose tissue were significantly higher in XOR+/− than in wild-type mice. Furthermore, histological analysis of epididymal white adipose tissue in XOR+/− mice revealed that adipocyte size and the F4/80-positive macrophage count were increased. Experiments with a high-fat diet exhibited that body weight gain was also significantly higher in XOR+/− than in wild-type mice. In stromal vascular fraction cells derived from XOR+/− mice, the levels of peroxisome proliferator–activated receptor γ, fatty acid–binding protein 4, and CCAAT enhancer–binding protein α mRNA were upregulated, and oxidative stress levels were elevated during differentiation into adipocytes.

Conclusions—These results suggest that the reduction in XOR gene expression in mice augments lipid accumulation in adipocytes, accompanied by an increase in oxidative stress, and induces obesity with insulin resistance in older age. (Arterioscler Thromb Vasc Biol. 2014;34:44-51.)

Key Words: adipose tissue ■ obesity ■ xanthine dehydrogenase

Xanthine oxidoreductase (XOR) is a key enzyme in purine catabolism, the process by which purine is converted to xanthine and uric acid.1 It has been reported that XOR is a critical source of reactive oxygen species (ROS) and nitric oxide and also plays an important role in a variety of physiological2 and pathophysiological conditions, such as ischemia–reperfusion injury,3 endothelial dysfunction in diabetes mellitus,4 and various cardiovascular diseases.5-9

Furthermore, XOR is a structural component of membrane-encapsulated milk fat globules,10 and mice lacking XOR have defects in fat droplet secretion.11 We also showed that XOR gene disruption induced the deletion of uric acid and the accumulation of triglyceride-rich substances and crystals in the renal tubules, with increased expression of adipogenesis-related genes such as peroxisome proliferator–activated receptor (PPAR) γ and the CCAAT enhancer–binding proteins (C/EBPs) β and C/EBPα.12 These findings suggest that XOR may be implicated in lipid metabolism and adipogenesis.

In a study on adipocyte differentiation using 3T3-L1 preadipocytes, it was reported that XOR lies downstream of C/EBPβ and upstream of PPARγ in the signaling pathway responsible for regulating adipocyte differentiation.13 Furthermore, Hallenborg et al14 have shown that XOR and epidermal-type lipoxygenase 3 may synergistically activate PPARγ and promote adipocyte differentiation through the activity of ROS produced by XOR. These studies using 3T3-L1 preadipocytes in vitro have suggested the possibility that decreased XOR activity may correlate with a reduction in lipid accumulation. However, the precise role of XOR in adipogenesis in vivo is not fully understood.

In this study, therefore, we used XOR heterozygous (XOR+/−) knockout mice to investigate the role of XOR activity in adipogenesis in vivo and also analyzed the effects of aging and intake of a high-fat diet (HFD) on the phenotype of XOR+/− mice. In addition, we examined adipogenesis using adipose tissue–derived stromal vascular fraction (SVF) cells...
prepared from XOR<sup>+/−</sup> mice and their wild-type (WT) littermates. The SVF cells are a heterogeneous cell population that includes adipocyte progenitor cells (preadipocytes).<sup>15,16</sup> Furthermore, preadipocytes in the SVF can differentiate into adipocytes by adequate hormonal stimulation. SVF cells, including macrophages and all components of adipose tissue except mature lipid-laden adipocytes, are closer to the physiological conditions of adipose tissue than 3T3-L1 cells. Thus, we used SVF cells prepared from XOR gene–disrupted mice to evaluate the role of XOR on adipogenesis in vitro.

**Materials and Methods**

Materials and Methods are available in the online-only Supplement.

**Results**

**Reduction in XOR Gene Expression Facilitates an Increase in Body Weight With Aging**

XOR<sup>+/−</sup> mice at 18 months of age showed significant increases in body weight (47.1±1.4 versus 39.4±0.6 g; *P*<0.05), epididymal fat pad weight (2.4±0.2 versus 1.4±0.1 g; *P*<0.05), and liver weight compared with WT mice without alteration of food intake (Figure 1A, 1C, and 1D and Figure I in the online-only Data Supplement). Systolic blood pressure was higher in XOR<sup>+/−</sup> mice than in WT mice (96.1±0.7 versus 90.7±0.9 mm Hg; *P*<0.05; Figure 1B). XOR<sup>+/−</sup> mice also showed significant increases in fasting blood glucose (145±4 versus 108±5 mg/dL; *P*<0.05), insulin concentrations (2.2±0.2 versus 1.5±0.2 ng/mL; *P*<0.05), and homeostasis model assessment of insulin resistance (20.4±1.8 versus 10.4±1.7; *P*<0.05) compared with WT mice (Figure 1F–1H), indicating obesity-associated impairment of glucose tolerance. However, the serum concentrations of cholesterol, triglyceride, and free fatty acids were not different between 18-month-old WT and XOR<sup>+/−</sup> mice (Table I in the online-only Data Supplement). The serum uric acid concentrations of XOR<sup>+/−</sup> mice were approximately half those of WT mice (Figure 1E). Inflammation of adipose tissue is associated with obesity-related insulin resistance. To assess the inflammation in epididymal white adipose tissue (eWAT), we performed quantitative reverse transcriptase-polymerase chain reaction analysis on the eWAT. XOR<sup>+/−</sup> mice showed increases in the expression of monocyte chemotactrant protein-1 (MCP-1), tumor necrosis factor α (TNF-α), and leptin mRNA and a decrease in the level of adiponectin mRNA, indicating that reduction in XOR expression caused the inflammation in eWAT (Figure 1I). XOR activity in eWAT at 2 and 12 months of age was 10- to 50-fold higher than in other tissues such as liver, kidney, and heart tissue (Figure IIA and IIB in the online-only Data Supplement), which suggested that XOR may play an important role in white adipose tissue.

**XOR<sup>+/−</sup> Mice at 4 Months of Age Exhibit Impaired Glucose Tolerance Without Obesity**

Phenotypic analysis of XOR<sup>+/−</sup> mice at 4 months of age showed that there were no differences in body weight and heart, kidney, and liver tissue weights, but the amount of eWAT was significantly greater in XOR<sup>+/−</sup> mice than in WT mice (Figure 2A and 2B). The area under the curve of blood glucose concentrations (2.4±0.3 versus 2.0±0.4 [mg/dL]×min×10<sup>4</sup>; *P*<0.05) in the intraperitoneal glucose tolerance test, fasting serum insulin (1.4±0.1 versus 1.1±0.1 ng/mL; *P*<0.05), and homeostasis model assessment of insulin resistance (11.5±1.4 versus 7.3±0.6; *P*<0.05) was increased in XOR<sup>+/−</sup> mice compared...
with WT mice (Figure 2C–2E). Systolic blood pressure was increased, and endothelial-dependent relaxation was attenuated in XOR+/− mice compared with WT mice (Figure 3). Serum concentrations of lipids were not different between XOR+/− and WT mice (Table I in the online-only Data Supplement). In addition, the expressions of C/EBPβ, PPARγ, fatty acid–binding protein 4 (FABP4), fat-specific protein 27, leptin, nicotinamide adenine dinucleotide phosphate oxidase 4 (Nox4), MCP-1, and TNFα mRNA were significantly increased in the eWAT of XOR+/− mice (Figure 2H). The oxidative stress level evaluated by the production of malondialdehyde and H2O2 in eWAT was also elevated in XOR+/− mice compared with WT mice (Figure 2F and 2G).

To assess the histological change, sections of eWAT were stained with hematoxylin and eosin. Enlarged adipocytes were found in XOR+/− mice compared with WT mice, and the average value of the adipocyte area was significantly greater in XOR+/− mice (3.45±0.04 versus 2.94±0.04 μm2×103; Figure 4A). Enlargement of the adipocytes releases higher amounts of proinflammatory mediators such as leptin and lower amounts of adiponectin. To quantify macrophage infiltration in the eWAT, immunohistochemical staining using the macrophage-specific marker F4/80 was performed. The percentage of F4/80-positive macrophages in XOR+/− mice was increased 5-fold compared with WT mice. Crown-like structures, which were associated with dead adipocytes surrounded by macrophages, were observed only in the eWAT of XOR+/− mice (Figure 4B).

High-Fat Diet Facilitates an Increase in Body Weight of XOR+/− Mice Compared With WT Mice
To investigate the influence of the XOR genotype on HFD-induced obesity, WT and XOR+/− mice at 2 months of age were fed a normal diet or HFD for 2 or 10 weeks. Body weight gain was similar for the 2 normal diet mouse groups, but between the 2 HFD groups, it was significantly increased in XOR+/− mice compared with the WT mice (45.9±0.7 versus 40.7±0.8 g; P<0.05; Figure 5A) without alteration of food intake (Figure III in the online-only Data Supplement). There were no differences in body or liver weight after 2 weeks with an HFD, but the amount of eW AT was significantly greater in XOR+/− HFD mice than in WT HFD mice (Figure 5B). Glucose tolerance evaluated by intraperitoneal glucose tolerance test was impaired, and fasting serum insulin levels (3.3±0.4 versus 1.8±0.3 ng/mL; P<0.05) and homeostasis model assessment of insulin resistance (31.3±4.7 versus 14.4±3.1; P<0.05) were increased in XOR+/− HFD mice compared with WT HFD mice (Figure 5C–5E). XOR+/− HFD mice showed increases...
in the expression of MCP-1, TNFα, and leptin mRNA and a decrease in the level of adiponectin mRNA (Figure 5F). In addition, enlarged adipocytes were detected in XOR+/− HFD mice compared with WT HFD mice, and the average value of the adipocyte area was significantly higher in XOR+/− mice (3.45±0.04 vs 2.94±0.04 μm²×10³). *P<0.05 compared with wild-type (WT) mice. These results suggest that XOR+/− mice facilitate obesity and insulin resistance by upregulation of fat accumulation in mature adipocytes.

SVF Cells Derived From eWAT in XOR+/− Mice Facilitate Adipocyte Differentiation

At 2 months of age, there were no differences in the body weight or eWAT weight between WT and XOR+/− mice. Insulin sensitivity indices, such as the glucose tolerance test, fasting insulin level, and homeostasis model assessment of insulin resistance, in XOR+/− mice were not different from those in WT mice (Figure 6A–6E). Furthermore, the mRNA expressions of the inflammation-related proteins MCP-1 and TNFα in the eWAT were not different between WT and XOR+/− mice, but the mRNA levels of adipocyte differentiation-related proteins such as C/EBPβ, PPARγ, and FABP4 were significantly higher in the eWAT of XOR+/− mice (Figure 6F). The production of H2O2 was significantly higher in XOR+/− mice, but the amount of malondialdehyde was not significantly different between the 2 groups (Figure 6G and 6H). We prepared SVF cells from these 2-month-old mice and evaluated their adipocyte differentiation. The SVF cells were differentiated into adipocytes, and accumulated lipids were stained with oil red O on day 7. Lipid accumulation in the differentiated SVF cells was much greater in XOR+/− mice than in WT mice (Figure 7A). The levels of PPARγ, FABP4, and C/EBPα mRNA, which normally increase during adipogenesis, were significantly increased in XOR+/− mice during adipocyte differentiation (Figure 7B). Interestingly, the expression of C/EBPβ mRNA was not induced by the stimulation of adipocyte differentiation, but it was significantly higher in XOR+/− mice than in WT mice during differentiation, suggesting that XOR+/− mice have sustained higher expression levels of C/EBPβ in adipose tissue. The SVF cells derived from XOR+/− mice showed a higher rate of H2O2 production compared with those from WT mice during differentiation (Figure 7C). Furthermore, the superoxide level was also significantly increased in XOR+/− mice compared with WT mice by nitroblue tetrazolium reduction assay (Figure 7D). To elucidate the source of ROS production, we examined 3 major pathways of ROS production, namely the XOR-, Nox4- and mitochondria-mediated pathways in the differentiated SVF cells. ROS production in differentiated...
SVF cells was not suppressed by oxypurinol, an inhibitor of XOR, or by rotenone, an inhibitor of mitochondrial electron transport chain complex I, but was suppressed by apocynin, an inhibitor of nicotinamide adenine dinucleotide phosphate oxidase (Figure 7E). In addition, the expression level of Nox4 mRNA was significantly higher in XOR+/− mice, but the mRNA expression levels of other Nox subunits in XOR+/− mice were not significantly different from those in WT mice (Figure 7B and Figure IV in the online-only Data Supplement). These results suggest that Nox4 is the major source of ROS in differentiated SVF cells and that the SVF cells derived from XOR+/− mice facilitate lipid accumulation in adipocytes exposed to higher oxidative stress during differentiation.

Discussion

In the present study, XOR+/− mice exhibited significantly increased body weight with aging and also showed higher rates of insulin resistance and higher blood pressure levels compared with WT mice. An HFD also induced significant gains in body weight and eWAT weight in XOR+/− mice compared with WT mice. The body weight in XOR+/− mice at 4 months of age was not different from that in WT mice; however, the eWAT in XOR+/− mice exhibited significantly increased body weight and insulin resistance and higher blood pressure levels compared with WT mice. XOR+/− mice at 2 months of age did not have inflammatory change, insulin resistance, or an increase in blood pressure but had higher expression levels of C/EBPβ, PPARγ, and FABP4 mRNA in SVF cells derived from XOR+/− mice. The expression of C/EBPβ mRNA in SVF cells derived from XOR+/− mice was not increased by the stimulation of adipocyte differentiation, but the level of this expression remained higher than in WT mice during differentiation.
These findings suggested that haploinsufficiency of XOR mice might induce sustained higher expression of C/EBPβ, adipocyte differentiation with exaggerated ROS production, and eventually obesity with insulin resistance in older age.

XOR−/− mice at 18 months of age showed obesity with an increase in the expression of MCP-1 mRNA in eWAT. In contrast, the level of adiponectin mRNA was decreased, and oxidative stress levels were upregulated in eWAT. These changes contribute substantially to obesity-related low-grade inflammation in eWAT. The fat accumulation in adipose tissue is associated with not only adipocyte hypertrophy but also an increase in the number of adipocytes. An increase in small adipocytes by adipogenesis usually improves insulin resistance through the production of adiponectin and leptin, whereas hypertrophy of adipocytes exacerbates insulin resistance.17 TNFα has been observed in the adipose tissue of obese mice with macrophage infiltration18,19 and has been shown to induce insulin resistance by phosphorylation of the serine residue of insulin receptor substrate-1.20 It has been reported that ROS production was markedly increased during differentiation of 3T3-L1 preadipocytes into adipocytes and in parallel with fat accumulation in adipocytes.21 In particular, regulated H$_2$O$_2$ production is an essential component of insulin signaling in adipose tissue.17,22,23 In this study, the SVF cells derived from XOR−/− mice exhibited higher levels of Nox4 expression and H$_2$O$_2$ production compared with those in WT mice during differentiation. XOR−/− mice at 4 months of age showed endothelial dysfunction in the aorta and an increase in systolic blood pressure and insulin resistance with no difference in body weight compared with WT mice. These mice had increases in adipose tissue weight, MCP-1, and TNFα mRNA levels and H$_2$O$_2$ in eWAT. Histological analysis revealed that eWAT of XOR−/− mice at 4 months of age exhibited a dramatic increase in F4/80-stained macrophage infiltration and an expansion of adipocyte size. These results suggest that the reduction in XOR gene expression exaggerated macrophage infiltration, TNFα expression, and H$_2$O$_2$ production, resulting in continuous low-grade inflammation and finally obesity with insulin resistance.

Obesity is characterized by increases in the number and size of adipocyte cells and the profound accumulation of triglycerides in adipose tissue. Fat accumulation is induced by an increase in energy intake or a decrease in energy consumption.24,25 In XOR−/− mice, the amounts of daily food intake and the levels of serum lipids were not different from those in WT mice, but the size of adipocytes was larger and their differentiation was faster than in WT mice. C/EBPβ is induced in the early phase of adipocyte terminal differentiation and lies upstream of PPARγ and C/EBPα in the cascade of adipogenesis. Activation of C/EBPβ induces transcription of both PPARγ and C/EBPα, which in turn coordinately activate metabolic genes such as leptin, FABP4, and adiponectin. In this study, C/EBPβ mRNA in the differentiated SVF cells showed a sustained higher expression in XOR−/− mice than in WT mice from day 0 to day 7, suggesting that a reduction in the XOR gene might consistently increase the expression of C/EBPβ mRNA and then induce the cascade of terminal adipocyte differentiation. In fact, there were no differences in body weight or eWAT weight, insulin sensitivity, or inflammation-related mRNA levels between the WT and XOR−/− mice at 2 months of age, but the mRNA expressions of adipocyte differentiation–related proteins, such as C/EBPβ, PPARγ, and FABP4, were significantly increased in XOR−/− mice compared with WT mice.

Recently, Chen et al26 reported that the amounts of plasma ceramide and spermidine in allopurinol-treated WT mice were the same as those in WT mice, although both compounds were significantly increased in the plasma of XOR−/− and XOR−/− mice. This result suggests that these 2 metabolites were not affected by XOR activity but by XOR gene expression. Ceramide and spermidine have been shown to be associated with fat accumulation.27,28 In addition, it has been reported that ceramide markedly induces the expression of C/EBPβ protein29 and that spermidine promotes C/EBPβ translation.30 Although we did not measure the plasma levels of ceramide and spermidine in the present study, these previous findings may support the idea that XOR influences the expression of C/EBPβ and regulates adipocyte differentiation through these metabolites.

The SVF cells derived from XOR−/− mice facilitated fat accumulation with higher oxidative stress. The expressions of PPARγ, FABP4, and C/EBPα mRNA were significantly increased in XOR−/− mice during differentiation. These results explain why the SVF cells prepared from XOR−/− mice showed enhanced adipocyte differentiation. In contrast, previous studies using 3T3-L1 cells or mouse embryonic fibroblasts have shown that the XOR gene increased PPARγ activity and adipocyte differentiation.13,14 It is difficult to account for the conflicting results between the present and previous studies. However, the divergence may be related to the experimental design, that is, previous studies used cells in which XOR gene expression was transiently inhibited, but we used haploinsufficient XOR mice that showed chronic inhibition of XOR gene expression from birth. These findings suggest that transient inhibition of the XOR gene in 3T3-L1 cells inhibits PPARγ activity and adipocyte differentiation, but chronic inhibition of the XOR gene accumulates plasma metabolites, such as ceramide and spermidine, and induces the sustained C/EBPβ expression, oxidative stress, and activation of PPARγ, probably because of an indirect effect of XOR expression in adipose tissue. Furthermore, it has been shown that the adipogenesis of adipogenic cell lines depends on an interplay among adipocyte precursors, vascular cells, and stromal cells.31 In addition, 3T3-L1 cells express the adipocyte-secreted factor leptin at much lower levels than primary adipocytes. White adipocytes comprise a single large droplet, but 3T3-L1 cells store triglycerides in many droplets.32 These results suggest that mouse cell lines may not reflect true adipocyte differentiation and real lipid metabolism in vivo.

XOR is a structural component of membrane-encapsulated milk fat globules and is necessary for milk fat droplet envelopment and secretion.10 The proteins belonging to the cell death–inducing DFF45-like effector family have been shown to associate with lipid droplets. Recently, Wang et al15 reported that Cidea, a cell death–inducing DFF45-like
effectors family protein, is expressed at high levels in lactating mammary glands, induces XOR expression by facilitating the association of C/EBPβ with the promoter of the XDH gene, and enhances lipid secretion in vivo. Furthermore, the fat-specific protein (also called Cidec), another cell death–inducing DFF45-like effector family protein, interacts with C/EBPβ to regulate the expression of XOR in adipocytes. In this study, the expression levels of both C/EBPβ and fat-specific protein mRNA in eWAT were significantly higher in 4-month-old XOR−/− mice than in WT mice of the same age. Different fat secretion mechanisms are at work in mammary epithelial cells and adipocytes; however, both the previous and present findings may indicate that XOR plays a pivotal role in adipocytes to regulate fat accumulation.

In conclusion, a reduction in XOR gene expression in mice induced fat accumulation and led to obesity with aging through increases in ROS, macrophage infiltration, and insulin resistance. Our study demonstrates that one of the roles of the XOR gene may regulate fat metabolism via C/EBPβ expression.

Acknowledgments

We appreciate the technical support from the Research Support Center, Graduate School of Medical Sciences, Kyushu University.

Sources of Funding

This work was supported, in part, by JSPS KAKENHI grant no. 23591195.

Disclosures

None.

References

Obesity is characterized by increases in the number and size of adipocyte cells and the profound accumulation of triglycerides in adipose tissue. Xanthine oxidoreductase (XOR) is an enzyme that catalyzes the final 2 steps in purine catabolism by converting hypoxanthine to xanthine and xanthine to uric acid, with concomitant generation of reactive oxygen species. In this study, XOR heterozygous (XOR+/−) mice showed enhanced lipid accumulation in adipocytes accompanied by an increase in oxidative stress, systolic blood pressure, insulin resistance, and the development of obesity in older age or with high-fat diet. Stromal vascular cells derived from XOR+/− mice differentiated into adipocytes with higher expression levels of peroxisome proliferator–activated receptor γ, fatty acid–binding protein 4, and CCAAT enhancer–binding protein α mRNA compared with XOR+/- mice. The expression of CCAAT enhancer–binding protein β mRNA in XOR+/− mice was sustained at a higher level compared with XOR+/- mice during adipocyte differentiation. XOR+/- mice provide a unique opportunity to study metabolic syndrome.
Mice Heterozygous for the Xanthine Oxidoreductase Gene Facilitate Lipid Accumulation in Adipocytes
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Arterioscler Thromb Vasc Biol. 2014;34:44-51; originally published online October 24, 2013; doi: 10.1161/ATVBAHA.113.302214
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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Supplemental Figure I. Body weights of XOR+/- mice at 2, 4, 12, 14 and 18 months of age compared with WT mice. Number of mice was showed in parenthesis. *P < 0.05 compared with WT mice.
Supplemental Figure II. XOR activity at 2 months (A) and 12 months (B) of age. XOR (XO and XDH) activity of tissue lysates from liver, eWAT, kidney and heart, n=5 per group.
Supplemental Figure III. Amount of daily food intake in four groups with ND or HFD, n=8 mice per group.
Supplemental Figure IV. Quantitative RT-PCR on SVF cells during post differentiation of WT and XOR⁺⁻ mice, n=6 mice per group. Data are normalized by the expression of TBP. Nox indicates NADPH oxidase.
**Supplemental Table I.** Serum uric acid and lipid variables at 4 and 18 months of age

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<td><strong>4 months</strong></td>
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<td>Total cholesterol (mg/dl)</td>
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<td>Uric acid (mg/dl)</td>
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n=8 per group
### Supplemental Table II. Sequences of real-time PCR primer pairs

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Materials and Methods

Animals

All animal procedures were conducted under protocols approved by the Committee on Ethics of Animal Experimentation of the Faculty of Medicine, Kyushu University. All mice used in the study were males, housed in a facility on a 12-h light / dark cycle with free access to food and water. XOR genetically modified mice were prepared as described previously and were the kind gift of Dr. Toren Finkel (NIH/NHLBI).

Intraperitoneal glucose tolerance test

A glucose tolerance test was performed after 12 h of fasting. Glucose (500 mg/kg) was injected intraperitoneally, blood was obtained from the tail, and the glucose level was measured at 0, 30, 60, 90 and 120 min after injection. The trapezoidal rule was used to determine the area under the curve (AUC) of blood glucose concentrations. Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated by using the following formula: HOMA-IR = 26 x fasting insulin level (ng/ml) x fasting glucose level (mg/dl))/405.

Lipid peroxidation

Epididymal white adipose tissue (eWAT) was homogenized in assay buffer (500 μl/50 mg tissue), then centrifuged at 15,000 rpm for 4 min at 4 °C. The supernatant was used for the assay. The levels of lipid peroxidation in eWAT homogenate were measured as a product of malondialdehyde (MDA) using an assay kit for thiobarbituric acid reactive substances (TBARS) (Northwest Life Science Specialties, LLC) according to the manufacturer’s instructions. Absorbance was measured using a Spectra Max M5 microplate reader (Molecular Devices, Sunnyvale, CA).

Hydrogen peroxide production in eWAT

Epididymal WAT was dissected out, placed in phosphate buffered saline (PBS), cut into
2-mm square pieces and incubated at 37 °C for 30 min. Hydrogen peroxide (H$_2$O$_2$) released from the tissue was detected using an Amplex Red Hydrogen Peroxide Assay Kit (Life Technologies Corp.).

**Blood pressure measurement**

Systolic blood pressure was measured with the tail-cuff method using a CODA monitor (Kent Scientific) in the unanesthetized state.

**Isometric tension recording**

Isometric tension was measured in ring segments of the descending thoracic aorta. The thoracic aortas of mice were cut into 2 mm rings and mounted in 5 mL organ chambers filled with modified Krebs solution aerated with 95% O$_2$ and 5% CO$_2$ at 36 °C. Two fine stainless-steel wires were placed through the lumen of the ring; one was anchored, and the other was attached to the mechanotransducer (UM-203; Kishimoto). The rings were stretched to 1 g of resting tension. After tissue equilibration, a control contraction in response to 77 mmol/L KCl was elicited. Cumulative dose-response curves for relaxation to acetylcholine and sodium nitroprusside were obtained in the rings pre-contracted with phenylephrine (10$^{-5}$ mol/L).

**Histological and immunohistological analysis**

Epididymal WAT was fixed for 72 h with 10% formaldehyde at 4°C, and then embedded in paraffin. Sections were cut at 4-μm thickness, mounted on glass slides, deparaffinized, rehydrated through a series of ethanol and washed in PBS.$^2$ For the measurement of adipocyte size, sections were stained with hematoxylin and eosin. Adipocyte sizes (100 adipocytes per sample) were calculated from cross-sectional areas obtained from perimeter tracings using Image J software (Sun Microsystems). For F4/80 staining, the glass slides were treated with 10 μg/ml proteinase K and 4N HCl. After blocking with 5% skim milk for 30 min at room temperature, the sections were incubated in a humidified chamber at 37°C for 1 h with anti-F4/80 antibody (1:500; AbD Serotec #MCA497R). Then the slides
were incubated with biotinylated anti-mouse IgG antibody, reacted with HRP-conjugated streptavidin and stained with DAB and H$_2$O$_2$. After counterstaining with hematoxylin, the slides were dehydrated and permanently mounted. Macrophage infiltration in adipose tissue was quantitated by calculating the ratio of nuclei of macrophage-positive cells to total nuclei in 5 fields of one slide for each of 4 mice in each group.

**High-fat diet feeding**

WT and XOR$^{+/−}$ mice at 2 months of age were fed either a normal (fat kcal 10%) or high-fat diet (fat kcal 56.7%) for 2 or 10 weeks.

**Isolation of the stromal vascular fraction from eWAT**

Epididymal WATs collected from WT and XOR$^{+/−}$ mice at 2 months of age were washed, cut into small pieces in PBS and incubated in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 0.5% collagenase type 1 and 1% bovine serum albumin (Wako Pure Chemicals Industries) at 37 °C for 60 min. Digested cells were passed through a 100-µm nylon mesh (BD Biosciences), followed by centrifugation at 1,500 rpm for 10 min. The SVF cells at the bottom were washed with PBS then centrifuged at 1,200 rpm for 10 min. The SVF pellet was resuspended in 2 ml of DMEM plus 10% FBS, seeded onto 35-mm collagen-coated dishes at a density of 4×10$^5$ cells/dish or 96-well plates at a density of 3×10$^4$ cells/well, and cultured at 37 °C under a humidified atmosphere of 95% O$_2$ + 5% CO$_2$.

**Induction of differentiation into adipocytes**

At 24-h after plating in DMEM plus 10% FBS, differentiation was induced by changing the medium to Visceral Adipocyte Culture Medium ver. 2 (Primary Cell Co., Ltd.). This differentiation medium was replaced every two days, and the cells were harvested on day 7 after the induction of differentiation.

**Oil Red O staining**

Oil Red O staining was performed to visualize the lipid accumulation in differentiating
adipocytes. SVF cells post-induction of adipocyte differentiation were fixed with 4% paraformaldehyde for 15 min, rinsed in PBS, and stained with Oil Red O (0.3% in 60% isopropanol) for 1 h. For quantification of Oil Red O, stained SVF cells seeded onto 96-well plates were dissolved with 99.5% isopropanol and their absorbance was determined at 520 nm using a microplate reader.

**ROS production in SVF cells**

H$_2$O$_2$ released from the SVF cells was detected using an Amplex Red Hydrogen Peroxide Assay Kit (Life Technologies Corp.). Superoxide production was detected by nitroblue tetrazolium (NBT) assay. NBT was reduced by ROS to a dark-blue, insoluble form of NBT called formazan. At 0, 2, 4, 6 and 8 days after induction of adipocyte differentiation, SVF cells seeded onto a 96-well plate were incubated for 90 min in PBS containing 0.2% NBT. Formazan was dissolved in 50% acetic acid, and its absorbance was determined at 560 nm using a microplate reader. The effects of three inhibitors, apocynin (Sigma-Aldrich #A10809), oxypurinol (Sigma-Aldrich #O6881) and rotenone (Sigma-Aldrich #R8875), on ROS production in SVF cells was examined at day 7 post-differentiation. One hour before the measurement of ROS production by NBT reduction assay, 200 μM apocynin, 100 μM oxypurinol or 100 μM rotenone was added to the culture medium.

**Xanthine oxidoreductase activity**

XOR activity was measured with a previously described fluorometric assay. The tissues were harvested and homogenized in xanthine dehydrogenase (XDH) assay buffer (50 mmol/L potassium phosphate, pH 7.4, 0.2 mmol/L ethylenediamine-$N,N,N',N'$-tetraacetic acid, and 1.0 mmol/L phenylmethylsulfonyl fluoride). The homogenates were centrifuged at 15,000 rpm at 4°C for 30 min. Xanthine oxidase (XO) enzyme activity was determined in a total volume of 200 μL consisting of 198 μL of the tissue sample and 2 μL pterin. XDH activity was measured as above but with the addition of 2 μL methylene blue. The
excitation frequency was 345 nm, and the emission wavelength was 390 nm. One unit of activity was defined as 1 μmol isoxanthopterin formed per minute, and XOR activity was determined as the sum of the measured XO and XDH activities.

**Quantitative RT-PCR**

Total RNAs from eWAT and SVF cells were prepared with an RNeasy Lipid Tissue Mini Kit (QIAGEN). Real-time RT-PCR was performed on an Applied Biosystems 7500 using a One-Step SYBR PrimeScript RT-PCR Kit II (Takara Bio Company) according to the protocol provided by the manufacturer. The sequences of the primers used in real-time PCR are shown in Supplemental Table II.

**Statistics**

The results are expressed as means ± SEM. The statistical significance of differences between the two groups was determined using Student’s t-tests. Values of $P < 0.05$ were considered significant.

**References**


