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REGULAR PAPER

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Mice derived from *in vitro* αMEM-cultured preimplantation embryos exhibit postprandial hyperglycemia and higher inflammatory gene expression in peripheral leukocytes

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ABSTRACT

We examined whether peripheral leukocytes of mice derived from *in vitro* αMEM-cultured embryos and exhibiting type 2 diabetes had higher expression of inflammatory-related genes associated with the development of atherosclerosis. Also, we examined the impact of a barley diet on inflammatory gene expression. Adult mice were produced by embryo transfer, after culturing two-cell embryos for 48 h in either α minimal essential media (α-MEM) or potassium simplex optimized medium control media. Mice were fed either a barley or rice diet for 10 weeks. Postprandial blood glucose and mRNA levels of several inflammatory genes, including *Tnfa* and *Nox2*, in blood leukocytes were significantly higher in MEM mice fed a rice diet compared with control mice. Barley intake reduced expression of *S100a8* and *Nox2*. In summary, MEM mice exhibited postprandial hyperglycemia and peripheral leukocytes with higher expression of genes related to the development of atherosclerosis, and barley intake reduced some gene expression.

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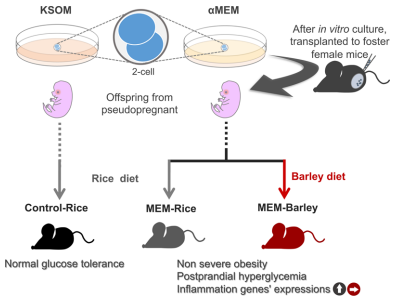
Graphical Abstract



Mice derived from αMEM culture during preimplantation exhibit postprandial hyperglycemia and higher peripheral leukocyte expression of inflammatory genes, and barley reduced expression of some genes.

Keywords: T2DM, *in vitro* fertilization, MEM mice, inflammation, barley

Abbreviations: αMEM: α minimal essential media; Cyc1: cytochrome c-1; CVD: cardiovascular disease; ICR: Institute of Cancer Research; IGT: impaired glucose tolerance; IL1B: interleukin 1B; IUGR: intrauterine growth restriction; KSOM: potassium simplex optimized medium; K-C: KSOM control mice; M-B: MEM mice fed a diet containing barley powder; MPO: myeloperoxidase; M-R: MEM mice fed a high rice powder diet; NOX: NADPH oxidase; OGTT: oral glucose tolerance test; ROS: reactive oxygen species; SEM: standard error of the mean; TNFA: tumor necrosis factor A; T2DM: type 2 diabetes mellitus

Clinical and epidemiological studies have indicated that undernutrition in the fetal and infant period can induce adult diseases in later life, called the developmental origins of health and disease (DOHaD) concept. A longitudinal study of adults born between 1952 and 1964 in famines in China reported that pregnant women exposed to the famine had an increased incidence of cardiovascular disease (CVD), which is one of the complications observed in type 2 diabetes mellitus (T2DM) patients, in their offspring (Zhang *et al.* 2019). Epidemiological studies have demonstrated that lower birth weight was positively associated with developing CVD and type 2 diabetes mellitus (T2DM) later in life (Tian *et al.* 2017; Zhao *et al.* 2018), noting that CVD is a known complication observed in patients with T2DM. In addition, *in vitro* fertilization may be associated with growth retardation and developing T2DM in later life (Chen, Norman and Heilbronn 2011). This evidence indicates that the fetal environment may contribute to the development of life-style diseases, in particular CVD and T2DM, in adulthood (Tian *et al.* 2017).

Animal models of fetal undernutrition have been used to study the pathology and mechanism of DOHaD. For example, fetal undernutrition by food restriction in pregnant rats caused a decrease in islet β-cell mass in the offspring (Garofano, Czernichow and Bréant 1998). In animal models of *in vitro* fertilization, 1 study reported that offspring had lower metabolic function, including impaired glucose tolerance (IGT) (Scott *et al.* 2010). However, this study has not assessed whether these mice exhibited T2DM and had a higher risk of CVD. Recently, we established that mice derived from embryos cultured in unbalanced nutrition α-MEM but not control media developed T2DM independently of obesity when fed a high-fat, high-sucrose diet after weaning (Kishigami, Mochizuki and Wakayama 2020).

Recent studies have suggested that postprandial hyperglycemia, rather than hemoglobin A1c, which indicates total glucose, is associated with the development of CVD. A 7-year cohort study in Japan demonstrated that subjects with IGT and patients with T2DM and postprandial hyperglycemia had a lower survival rate from CVD than healthy subjects (Tominaga *et al.* 1999). The development of CVD by chronic hyperglycemia is thought to occur by the following mechanism. Hyperglycemia activates the glycolytic pathway and related pathways, such as the tricarboxylic acid cycle and pentose phosphate pathway, leading to the production of reactive oxygen species (ROS) in mitochondria (Niedowicz and Daleke 2005; Yan 2014) by ROS producing enzymes, such as NADPH oxidase (NOX), xanthine oxidase and myeloperoxidase (MPO) (Niedowicz and Daleke 2005; Förstermann 2010). ROS activates innate leukocytes and induces secretion of proinflammatory cytokines (Domingueti *et al.* 2016). Proinflammatory cytokines, such as interleukin 1B (IL1B) and tumor necrosis factor A (TNFA), stimulate vascular endothelial cells to induce expression of adhesion molecules (Majewska *et al.* 1997; Pober 2002), which promote leukocyte adhesion to the endothelium, resulting in atherosclerosis (Galkina and Ley 2009). Recent studies have demonstrated increased expression of proinflammatory cytokines (*Il1b* and *Tnfa*) and the S100 proteins (*S100a4*, *6*, *8*, and *9*), which enhance expression of proinflammatory cytokines, in peripheral leukocytes of streptozotocin diabetic rats (Tanaka *et al.* 2009) and in T2DM patients (Osonoi *et al.* 2010). This induction of gene expression was reduced by treatment with the α-glucosidase inhibitor miglitol, which inhibits postprandial hyperglycemia (Fukaya *et al.* 2009). These results suggest that repeated postprandial hyperglycemia induces the expression of proinflammatory genes in peripheral leukocytes and could eventually lead to atherosclerosis-related diseases, including CVD.

In this study, we determined whether mice derived from *in vitro* αMEM-cultured preimplantation embryos (MEM mice) exhibited postprandial hyperglycemia and had higher expression of genes related to inflammation in peripheral leukocytes. We also investigated whether the administration of barley in the diet, which can reduce postprandial hyperglycemia (Matsuoka *et al.* 2020), provided an effective treatment for reducing inflammatory gene expression in peripheral leukocytes.

Materials and methods

Animals

For the generation of MEM and KSOM mice, two-cell stage embryos were collected from oviducts 48 h after mating and cultured in either α minimum essential medium (α-MEM; 135-15175, Wako Pure Chemical Industries, Ltd., Osaka, Japan) or potassium simplex optimized medium (KSOM) (ARK Resource, Kumamoto, Japan) control medium for 48 h. Details of both media are shown in Table S1. Embryos were transferred into oviducts of pseudopregnant recipients. The culture method for generating mice was based on a previously reported method (Boiani *et al*. 2005). All animals were maintained at 2 mice per cage under controlled conditions (temperature 23 ± 2 °C; humidity 50 ± 0%; 12-h light/12-h dark cycle). From 10 to 16 weeks of age, the mice were fed a westernized diet, which included high sugar and high fat, for 58 days. A patent for producing a T2DM animal model by culturing two-cell stage embryos in α-MEM and a subsequent high fat/high sugar diet after weaning was accepted (Kishigami, Mochizuki and Wakayama 2020). Twenty-four male MEM mice and 8 male KSOM mice, which were produced from (Institute of Cancer Research) (ICR) strain, (19-25 weeks of age) were transferred from Kiwa Laboratory Animals Co., Ltd. (Wakayama, Japan) to the University of Yamanashi and given food and water ad libitum. Body weights and blood glucose concentrations after 3 h of fasting were measured for all animals. MEM mice were then divided into 2 groups of similar age, body weight and 3-h fasting blood glucose concentrations. Three groups were started on the following test diets: (1) KSOM control mice ($n = 8$, K-C) fed a diet containing high rice powder (Niigata Flour Milling Co., Ltd., Niigata, Japan); (2) MEM mice fed a high rice powder diet ($n = 12$, M-R); (3) MEM mice fed a diet containing barley powder provided by Hakubaku Co., Ltd. (Yamanashi, Japan; $n = 12$, M-B). One mouse in the M-B group died during the oral glucose tolerance test (OGTT); 8, 12, and 11 mice in the K-C, M-R group, and M-B groups, respectively, completed the test diets; and body weights, food intake, organ weights and blood parameters were analyzed. Just before starting the test diets, the average ages of K-C, M-R, and M-B mice were 19 ± 0.0, 21 ± 0.6, and 21 ± 0.7 (means ± SEM) weeks old, respectively. Diets were ordered from Oriental Yeast Co., Ltd. (Tokyo, Japan), and their composition is shown in Table 1.

The barley diet included soluble fiber β-glucan (average of 1.06 g/100 g barley, $n = 2$), as determined by Hakubaku Co., Ltd. Body weights and food intake were measured every 2 weeks. This study was approved by the Animal Ethics Committee at the University of Yamanashi and was performed according to institutional animal experiment regulations (Approval number A30-24).

Table 1. Formulation of experimental diets (g/kg)

	Western	Rice diet	Barley diet
Casein	198.2	198.2	198.2
L-Cysteine	3	3	3
β-Corn starch	37.458	–	–
α-Corn starch	12.5	150	150
Sucrose	340	99.8	99.8
Maltodextrin	99.8	–	–
Rice powder ingredient	–	239.958	119.958
Barley powder ingredient	–	–	120
Soybean oil	10	10	10
Unsalted butter	200	200	200
Cholesterol	1.5	1.5	1.5
Cellulose powder	50	50	50
AIN93G–mineral mix	35	35	35
AIN93G–vitamin mix	10	10	10
Choline bitartrate	2.5	2.5	2.5
t-Butylhydroquinone	0.042	0.042	0.042
Total (g)	1000	1000	1000
kcal/kg	4183	4233	4199

Oral glucose tolerance test

The OGTT was performed after 6 h fasting at 9 weeks of diet administration, and times of glucose oral loading, measurement of blood glucose concentration, and blood collection in mice were adjusted to be equivalent for all groups. After a glucose load (2 g/kg body weight), blood was collected from the tail vein of all mice at 0, 15, 30, 60, and 120 min using a capillary tube with heparin (#02-668-10, Thermo Fisher Scientific Inc, Waltham, MA). The samples were centrifuged, and supernatants containing plasma were collected and stored at −80 °C until use. Blood samples for RNA analysis were collected just before and 120 min after glucose loading in PAXgene® Blood RNA tubes (Becton, Dickinson and Company, Franklin Lakes, NJ). The samples for RNA analysis were allowed to stand at room temperature for 24 h and then stored at −80 °C until use.

Dissection and sample collection

Dissections were performed between 10.00 am to 2.00 pm under nonfasting conditions at 10 weeks after the start of the test diets, and average dissection times were adjusted to be similar for all groups by assignment of dissection times. After measuring body weights, mice were decapitated and then blood was collected from the carotid artery using tubes containing heparin (CJ-2HL, TERUMO Corporation, Tokyo, Japan). Blood glucose was measured using a self-measuring device (Glutest Mint, Sanwa Kagaku Kenkyusho Co. Ltd, Aichi, Japan). Samples were collected for plasma and RNA analysis and stored as above.

Determination of plasma insulin and triglyceride concentrations

The plasma insulin concentrations were determined with an LBIS Mouse Insulin ELISA Kit (AKRIN-011T, FUJIFILM Wako Shibayagi, Gunma, Japan). Plasma triglyceride concentrations were quantified using a Triglyceride E Test Wako Kit (432-40201, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). Each test was performed following the manufacturer's protocol.

Quantitative real-time RT-PCR

Total RNA was extracted from blood with a RNeasy mini-Kit (Qiagen K.K., Hilden, Germany) and quantified using a Qubit™ Assay Kit (Thermo Fisher Scientific Inc). Total RNA was converted into cDNA using SuperScript III reverse transcriptase (Thermo Fisher Scientific Inc), according to the manufacturer's instructions. Quantitative real-time RT-PCR (qRT-PCR) was conducted using LightCycler 480 Universal Probes Master (NIPPON Genetics Co, Ltd., Tokyo, Japan) and a LightCycler System (Roche Diagnostics K.K., Basel, Switzerland), following the manufacturer's protocols. The primer sequences and Universal Probe Library numbers are shown in Table 2. The ratio between the

Table 2. The sequence of oligonucleotide primers used for real-time RT-PCR

Target mouse mRNA	Sequence	Universal probe no.
Catalase (*Cat*)	5′-TGAAGGATCCTGACATGGTCT-3′ 5′-CCTCGGTCACTGAACAAGAAA-3′	109
CD11a (*Cd11a*)	5′-ATTTTCCTGGCGCTCTACAA-3′ 5′-CTTCTGGAGGGCTTCCATTT-3′	75
CD11b (*Cd11b*)	5′-TATTTGTTCGGCTCCAACCT-3′ 5′-TCTCCTGCTGAGGACATTCTCT-3′	3
CD11c (*Cd11c*)	5′-TCTGGCAGATGTGGCTATTG-3′ 5′-TCTGGGATGCTGAAATCCTC-3′	55
CD18 (*Cd18*)	5′-AATGCAAGTTGCTGCATGTC-3′ 5′-CAAAGTGGAAGCCATCGTCT-3′	66
Cytochrome c-1 (*Cyc1*)	5′-TGCTACACGGAGGAAGAAGC-3′ 5′-CCATCATCATTAGGGCCATC-3′	10
Interleukin-1 beta (*Il1b*)	5′-TCTTCCTAAAGTATGGGCTGGA-3′ 5′-AAAGGGAGCTCCTTAACATGC-3′	60
Myeloperoxidase (*Mpo*)	5′-CTGAATCCTCGATGGAATGG-3′ 5′-CCATGGCCCCTACAATCTT-3′	79
NADPH oxidase subunit (47 kDa) (*p47phox*)	5′-GGACACCTTCATTCGCCATA-3′ 5′-CTGCCACTTAACCAGGAACAT-3′	3
NADPH oxidase subunit (67 kDa) (*p67phox*)	5′-TCCACCACCTCCTAATTCTAGC-3′ 5′-GGCTCTTTTTGCTTGTGACC-3′	109
NADPH oxidase 2 (*Nox2*)	5′-TGCCAACTTCCTCAGCTACA-3′ 5′-GTGCACAGCAAAGTGATTGG-3′	20
Rac family small GTPase 1 (*Rac1*)	5′-AGATGCAGGCCATCAAGTGT-3′ 5′-GAGCAGGCAGGTTTTACCAA-3′	77
Superoxide dismutase 1, soluble (*Sod1*)	5′-CAGGACCTCATTTTAATCCTCAC-3′ 5′-TGCCCAGGTCTCCAACAT-3′	49
Superoxide dismutase 2, soluble (*Sod2*)	5′-TGGACAAACCTGAGCCCTAA-3′ 5′-GACCCAAAGTCACGCTTGATA-3′	67
S100 calcium binding protein A4 (*S100a4*)	5′-TGCATTCCAGAAGGTGATGA-3′ 5′-CTCCTGGAAGTCAACTTCATTGT-3′	56
S100 calcium binding protein A6 (*S100a6*)	5′-ACTCTGGCAAGGAAGGTGAC-3′ 5′-CAATGGTGAGCTCCTTCTGG-3′	17
S100 calcium binding protein A8 (*S100a8*)	5′-TGCGATGGTGATAAAAGTGG-3′ 5′-GGCCAGAAGCTCTGCTACTC-3′	5
S100 calcium binding protein A9 (*S100a9*)	5′-AATGGTGGAAGCACAGTTGG-3′ 5′-CTGATTGTCCTGGTGCTCAG-3′	85
S100 calcium binding protein A10 (*S100a10*)	5′-CCTCTGGCTGTGGACAAAAT-3′ 5′-AAGCCCACTTTGCCATCTC-3′	85
S100 calcium binding protein A11 (*S100a11*)	5′-CCCAGAAGCGAATCTAATCCT-3′ 5′-AGGCGTGGGATACATGTTGT-3′	107
Tumor necrosis factor alpha (*Tnfa*)	5′-TCTTCTCATTCCTGCTTGTGG-3′ 5′-CACCCCGAAGTTCAGTAGACA-3′	49
Xanthine dehydrogenase (*Xdh*)	5′-TTGCATCATGTTGCTGTGAC-3′ 5′-TGGCTTTTGGCAATTCTCTC-3′	97

[a]The numbers in parentheses indicate ID of a universal probe (Roche Diagnostics) used to detect the signals of each gene.

mRNA expression level of the target gene and the house keeping cytochrome c-1 gene (*Cyc1*) control was calculated, and the relative values were determined as described in a previous report (Livak and Schmittgen 2001). A sample from the M-B group was removed because the cDNAs were not suitably amplified (Ct value from qPCR for *Cyc1* was 4.6-fold greater than the *Cyc1* mean, beyond the mean ± 2 SD). Therefore, we analyzed qRT-PCR using samples from 8, 12, and 10 mice from the K-C, M-R, and M-B groups, respectively.

Statistical analysis

All experimental data are presented as the mean ± SEM. In this study, we compared data from mice as 2 statistic models, such as different culture media (α-MEM or KSOM) or different diets (rice or barley). The unpaired Student's *t*-test was used to compare K-C and M-R groups, and M-R and M-B groups. Paired Student's *t*-test was performed for comparisons between 0 and 120 min after oral glucose loading in each group. The differences between groups were considered statistically significant when $P < .05$.

Results

Body weights and food intake of M-R, M-B, and K-C mice

Before administration of test diets (rice or barley), body weight was greater in M-R than in K-C mice, but no differences were observed between the M-R and M-B groups (Table 3). Blood glucose levels at the start of diet administration were equivalent between M-R and K-C mice, and between M-R and M-B mice. At 10 weeks after diet administration, no differences in body weight

Table 3. Effects of a 10-week barley diet on body composition and food consumption in mice

		αMEM	
	KSOM	Rice diet	Barley diet
Body weight at administration of test diets (g)	58.9 ± 1.2	75.0 ± 2.9*	75.1 ± 3.3
Body weight at OGTT (g)	70.9 ± 4.1	81.8 ± 4.4	85.4 ± 4.0
Body weight after 10 weeks (g)	70 ± 3.5	81 ± 4.2	84 ± 3.8
Blood sugar at administration of test diets (mg/dL)	143 ± 10	256 ± 48	259 ± 43
Plasma triglyceride (mg/dL)	179 ± 17	184 ± 27	199 ± 22
Food consumption (g/day)	4.74 ± .04	7.22 ± 0.46*	8.56 ± 1.08

*Significantly different from KSOM control mice ($^*P < .05$).
Data are expressed as means ± SEM for KSOM control mice ($n = 8$), and MEM mice fed a rice powder diet ($n = 12$) and those fed a barley diet ($n = 11$) (19-21 weeks of age). Statistical analyses were performed by the Student's t-test.
Abbreviation: OGTT, oral glucose tolerance test.

were observed between M-R and K-C mice, or between M-R and M-B mice. Food intake per day during the experimental period was higher in M-R than in K-C mice but did not differ between M-R and M-B mice.

Blood glucose and plasma insulin concentrations after administration of test diets

Blood glucose concentrations at 15 min after oral glucose loading were higher in M-R than in K-C mice (Figure 1a). However, at 0 (fasting), 30, 60, and 120 min after oral glucose loading, there were no differences in blood glucose levels between K-C and M-R mice or between M-R and M-B mice. Plasma insulin concentrations were similar between K-C and M-R mice, and between M-R and M-B mice at all time points tested.

Nonfasting blood glucose concentrations before administration of the test diet tended to be higher in M-R than in K-C mice ($P = .06$) but were similar between M-R and M-B mice (Figure 1b).

At 10 weeks after administration of the test diet, nonfasting blood glucose and insulin concentrations were similar between K-C and M-R mice or between M-R and M-B mice (Figure 1c). Nonfasting plasma insulin concentrations at the period tended to be lower in M-B than in M-R ($P = .08$).

mRNA expression of inflammatory genes in peripheral leukocytes of M-R, M-B, and K-C mice

At 9 weeks, fasting mRNA levels for the inflammatory markers *Il1b, Tnfa*, *Cd11a*, *Cd11b*, *Cd11c*, *S100a4*, *S100a6*, *S100a8*, *S100a9*, *S100a10*, *S100a11*, and *Cd18* did not differ between K-C and M-R mice or between M-R and M-B mice (Figure 2). At 120 min after oral glucose loading, mRNA levels of *Tnfa*, *Cd11a, Cd11b*, and *Cd18* in M-R mice were significantly higher compared with K-C mice. The mRNA expression of genes related to inflammation at 120 min after oral glucose loading did not differ between M-R and M-B mice.

At 10 weeks, fasting mRNA levels of *Il1b*, *Cd11b, Cd11c, S100a6*, and *Cd18* was greater in M-R than in K-C mice, and the mRNA levels of *Tnfa* was higher in M-R than in K-C mice, but this was not significant ($P = .052$; Figure 3). In MEM mice, *S100a8* mRNA levels were lower in M-B than in M-R groups, and mRNA levels of *S100a6* and *S100a9* in M-B mice tended to be lower compared with those in M-R mice ($P = .08$ and $P = .07$, respectively).

mRNA expression levels of oxidative stress genes in peripheral leukocytes of M-R, M-B, and K-C mice

At 9 weeks, the fasting mRNA levels of *Nox2* were higher in M-R than in K-C mice, and this increase in *Nox2* was reduced in M-B compared with M-R mice (Figure 4a). The mRNA levels of *Cat* tended to be lower in M-R mice compared with K-C mice ($P = .08$).

At 120 min after oral glucose loading, *Nox2* mRNA expression was higher in M-R compared with K-C mice, and this was slightly reduced in M-B compared with M-R mice ($P = .09$) (Figure 4b). At 120 min after oral glucose loading, *p47phox*, *p67phox*, and *Rac1* mRNA levels were higher in M-R than in K-C mice. The mRNA levels of *Cat* tended to be lower in M-R than in K-C mice ($P = .06$). No differences in *Sod1*, *Sod2*, *Xdh*, and *Mpo* mRNA levels were observed between K-C and M-R or between M-R and M-B groups at any time point during OGTT.

At 10 weeks, *Sod1*, *Sod2*, *Nox2*, *Xdh*, *Mpo*, *p47phox*, and *p67phox* mRNA levels were higher in M-R than in K-C mice (Figure 5). *Cat* expression tended to show a higher level in M-R compared with K-C mice ($P = .06$).

Comparison of peripheral leukocyte mRNA expression levels of inflammatory and oxidative stress genes between 0 min (fasting) and 120 min glucose loading for each group

Increased *Cd18* and *S100a4* mRNA expression levels at 120 min compared with 0 min were observed in M-B mice. Decreased *Tnfa* mRNA expression levels at 120 min versus 0 min were observed in K-C mice (Table S2).

Discussion

In this study, we have demonstrated that MEM mice, derived from preimplantation embryos cultured in α-MEM, showed moderate weight gain and hyperglycemia at fasting and postprandial periods for 9 weeks after administration of the test diet and higher expression of inflammatory genes in peripheral leukocytes. Although the precise mechanism and contributing factors remain unknown, this study shows that the preimplantation α-MEM environment results in the production of a nonobese T2DM mouse model, which shows postprandial hyperglycemia.

Many past studies of DOHaD using rodent models have included a surgical approach using ligation of the uterine artery or food restriction in the gestational/weaning period. These models showed that a disbalance of the fetal nutrient environment

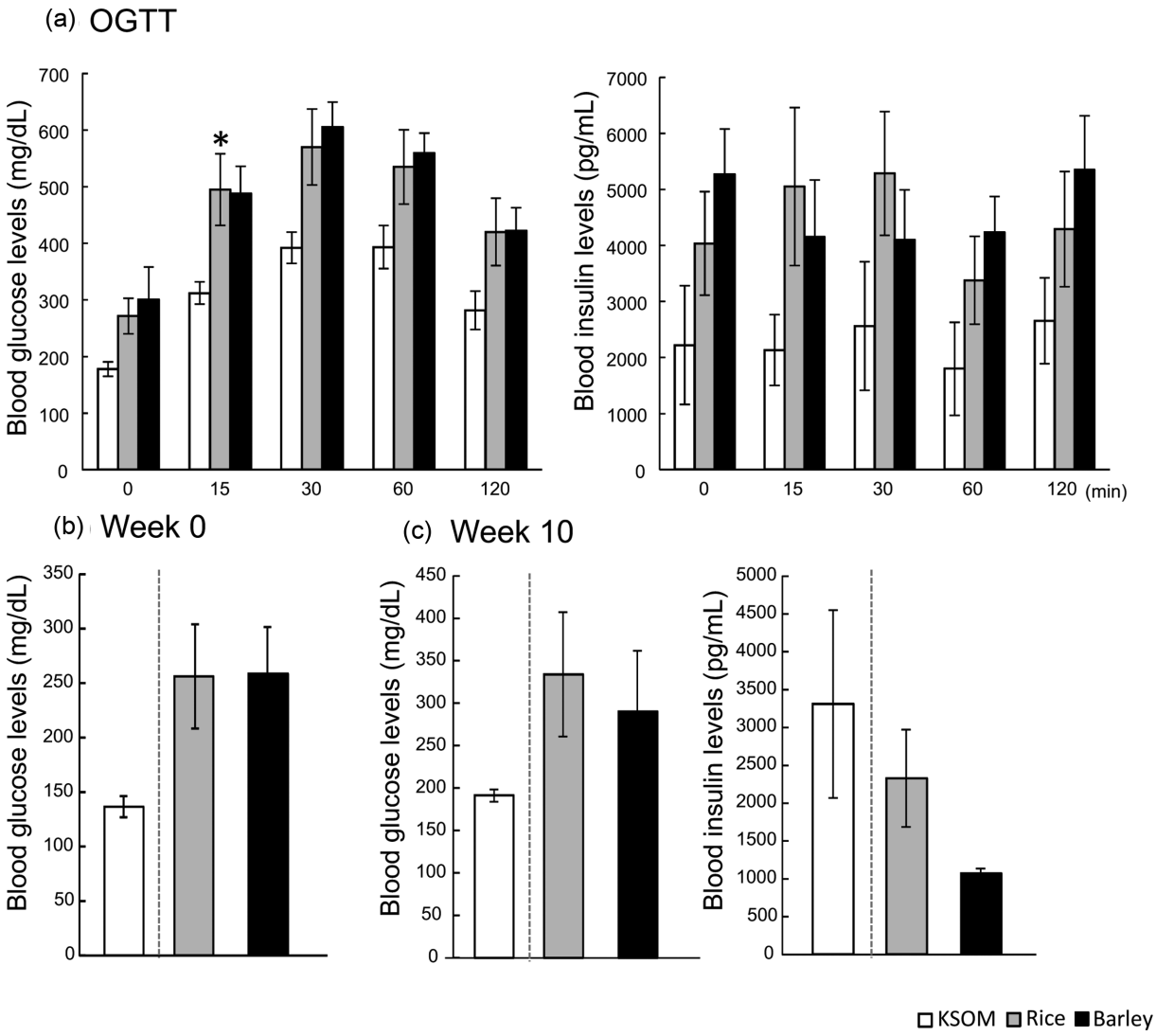


Figure 1. Blood glucose and plasma insulin concentrations in the fasted state, after oral glucose loading and in the nonfasted state in MEM mice fed a barley powder diet (M-B; $n = 11$) or a high-rice powder diet (M-R; $n = 12$), and KSOM control mice (K-C; $n = 8$). Data are expressed as the mean ± SEM for 8-12 animals. The data were analyzed by a Student's *t*-test. Asterisks (*) denote significant differences from K-C, where $P < .05$.

induced a fatty liver (Deodati *et al.* 2018) and IGT (Jin *et al.* 2019). Our study is the first to demonstrate that an embryo nutrient environment produced by *in vitro* conditions induced T2DM in MEM mice, in particular, higher postprandial hyperglycemia without excessive weight gain. However, our study did not monitor the course of progression of T2DM by measuring glucose and insulin concentrations during the development of MEM mice. We are currently carrying out these studies and monitoring body weight, glycemic control and blood insulin levels in MEM mice fed a high-fat, high-sucrose diet from birth to 30 weeks of age (Kishigami, Mochizuki and Wakayama 2020).

Our present study also demonstrated that mRNA expression of several inflammatory genes, such as *Cd11a*, *Cd11b*, *Cd11c*, *Cd18*, *Il1b*, *S100a6*, and *Tnfa*, in particular during the postprandial period, was induced in MEM mice fed a rice powder diet compared with KSOM control mice. These inflammatory-related genes encoded proinflammatory cytokines, which are secreted by leukocytes and are part of the early immune response (*Il1b* and *Tnfa*) (Lopez-Castejon and Brough 2011), adhesion molecules, such as integrins, which bind the vascular endothelium (*Cd11a*, *Cd11b*, *Cd11c*, and *Cd18*) (Schittenhelm, Hilkens and Morrison 2017), S100 protein family members that promote inflammatory cytokine expression (*S100a8* and *S100a9*) (Wang *et al.* 2018), and a gene inducing ROS production (*S100a6*). A recent animal study reported that 50% food restriction, which is a typical DOHaD model, induced higher *Cd11b* mRNA levels at 180 min after glucose loading than at fasting (Jin *et al.* 2019). Elevated *Cd11b* expression in our MEM mouse model is consistent with this previous study.

Past reports indicated that postprandial hyperglycemia is positively associated with the subsequent development of CVD (Laakso 1999; Bonora and Muggeo 2001). In addition, a randomized and double-blind trial for patients with a history of myocardial infarction found that the hazard ratio of cardiovascular events in patients receiving a therapeutic dose of antibody targeting IL1B was 0.85 (95% CI, 0.74-0.98; $P = .021$) compared with placebo, at a median follow-up of 3.7 years (Ridker *et al.* 2017). In our study, the expression of proinflammatory genes encoding the ROS-producing enzyme NADPH oxidase, including the membrane component *Nox2* (Paravicini and Touyz 2008)

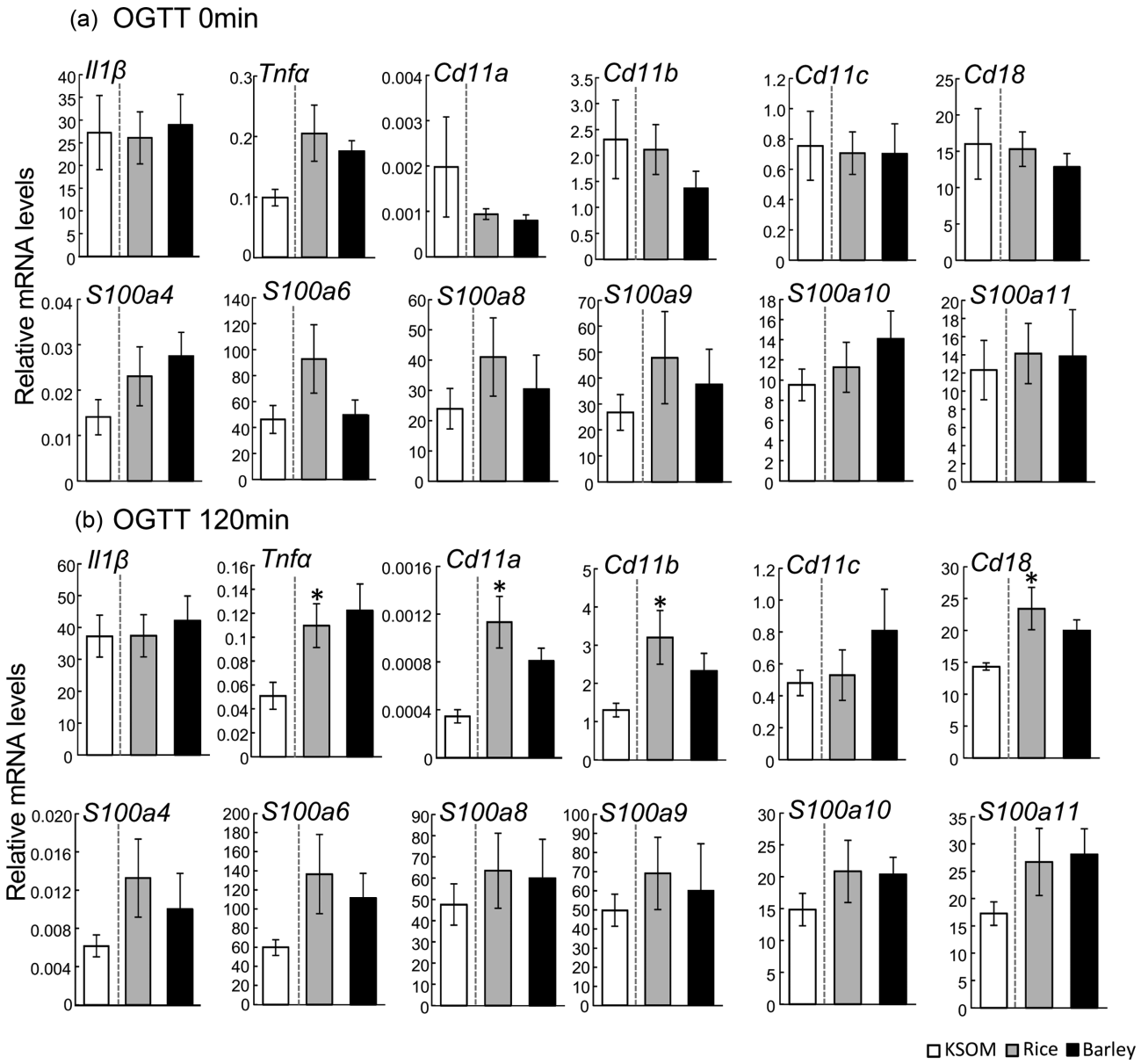


Figure 2. mRNA levels of inflammatory genes in peripheral leukocytes in the fasted state or after oral glucose loading in MEM mice fed a barley diet group (M-B; $n = 10$), those fed a high-rice powder diet group (M-R; $n = 12$), and KSOM control mice (K-C; $n = 8$). Data are expressed as the mean ± SEM for 8-12 animals. The data were analyzed by a Student's *t*-test. Asterisks (*) denote significant differences from K-C, where $P < .05$.

and cytosolic components (*p47phox*, *p67phox*, and *Rac1*) (Elnakish *et al.* 2013), were higher in peripheral blood leukocytes of MEM mice fed a rice powder diet at nonfasting and postprandial hyperglycemic periods than in KSOM control mice. It has been reported that the expression levels of NADPH oxidase, including *Nox2*, *p22phox*, and *p40phox*, were higher in the coronary artery of *db/db* mice than in that of control mice (Gao *et al.* 2008). Similarly, it was reported that *p22phox* gene expression in peripheral blood leukocytes was higher in patients with T2DM, including those with microvascular impairment, than in healthy subjects (Adaikalakoteswari *et al.* 2006). Considering these previous findings, our data suggest that the MEM diabetic mouse model had a higher inflammatory response, which was associated with increased oxidative stress and NADPH oxidase activity, compared with KSOM control mice. However, the expression of other genes, for example *S100a4* was not higher in MEM mice fed a rice powder diet than in KSOM control mice. Therefore, the higher expression of several inflammatory genes in peripheral leukocytes of MEM mice may be caused by the development of T2DM. Further study is required to investigate the causes of T2DM in MEM mice, and to determine whether the expression of inflammatory genes in peripheral leukocytes was induced by the development of T2DM. The *db/db* mice exhibit a mutation in the leptin receptor and develop severe obesity, so are considered a typical T2DM model with severe obesity (Genuth, Przybylski and Rosenberg 1971; Dubuc 1976). Our current results provide evidence that inflammatory responses in peripheral leukocytes are present in diabetic animal models even with moderate hyperglycemia and without severe obesity, such as MEM mice, as well as in models with severe hyperglycemia and obesity, such

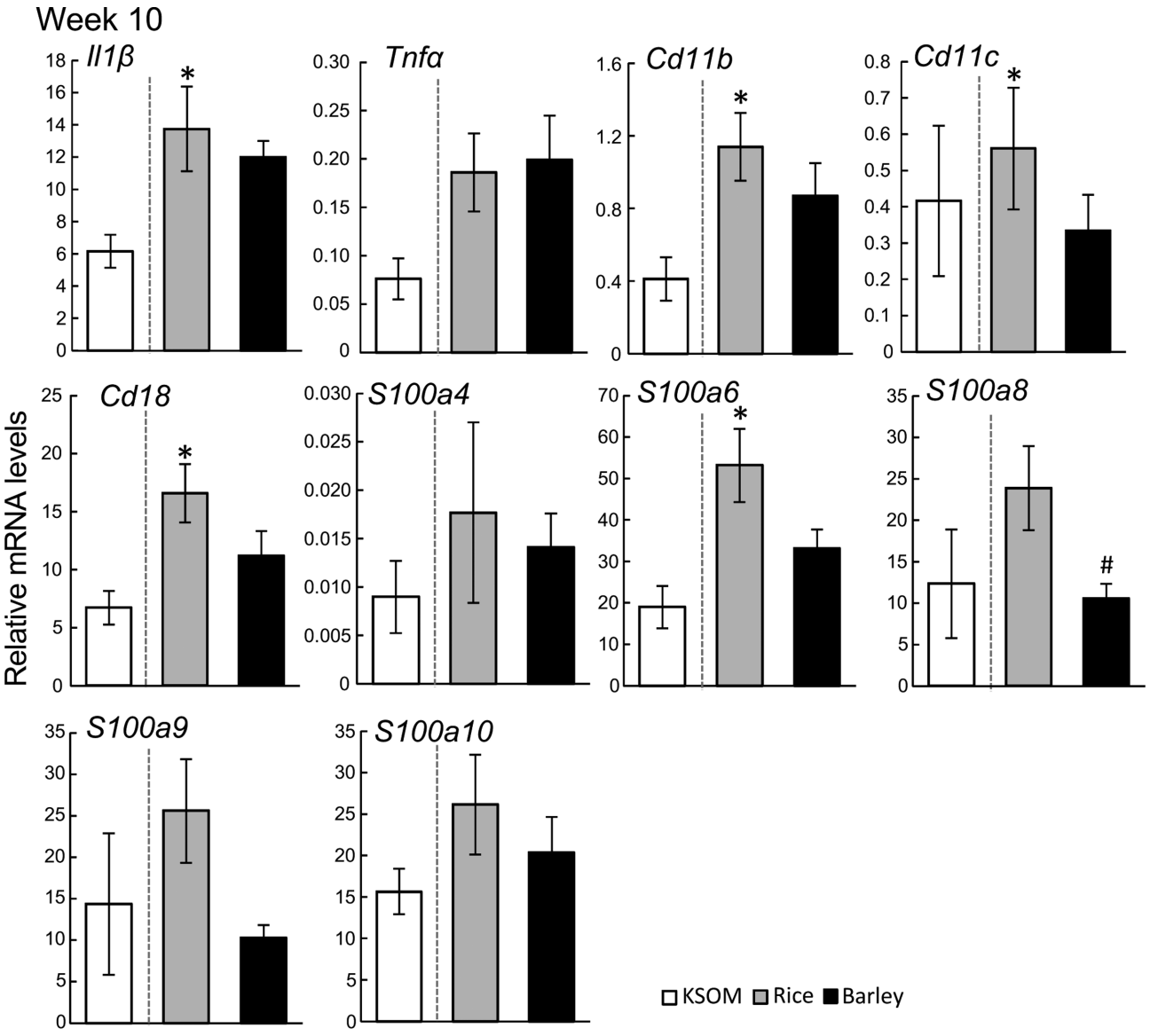


Figure 3. mRNA levels of inflammatory genes in peripheral leukocytes 10 weeks after administration of test diets. MEM mice were either fed a barley diet (M-B; $n = 10$) or a rice diet (M-R; $n = 12$), and KSOM control mice (K-C; $n = 8$) were fed a rice diet. Data are expressed as the mean ± SEM for 8-12 animals. Data were analyzed by a Student's *t*-test. Asterisks (*) denote significant differences from K-C mice, where $P < .05$. The pound sign (#) denotes significant differences from M-R mice, where $P < .05$.

as *db/db* mice. Therefore, we propose that MEM mice provide a suitable nonobese T2DM model to investigate the development of atherosclerosis. Further studies are needed to determine whether MEM mice exhibit atherosclerosis-related diabetic complications, including CVD, nephropathy, and retinopathy, and whether these pathologies are suppressed by barley intake.

In the present study, we found that a barley powder diet reduced *S100a8* expression after 10 weeks barley administration and decreased *Nox2* expression in peripheral leukocytes of fasting MEM mice. It is known that S100A8 induces proinflammatory cytokines by activating the receptor of advanced glycosylation end products (Cohen 2013), and NOX2 induces proinflammatory cytokines by activating NF-κB (Li *et al*. 2018). These results indicate that barley may have a protective role and reduce inflammation in MEM mice. Additionally, we note that *Nox2* and *S100a8* expression levels in peripheral leukocytes were reduced by feeding MEM mice a barely powder diet, despite no observed improvement in glycemic control by this diet. A possible explanation is that daily decreases in postprandial glucose concentrations, which cannot be assessed by nonfasting blood glucose concentrations at 10 weeks after test diet administration, reduced the expression of these genes in peripheral leukocytes. Another possibility is that barely intake affects gut microbiota populations and such changes impact the expression of inflammatory genes in peripheral leukocytes. Indeed, several studies indicate that dietary fiber in foods, including barely, alter gut microbiota populations in human and mice. For example, it was reported that a 3-day consumption of barley kernel-based bread affected the abundance of the *Prevotella* ratio in fecal microbiota of healthy subjects (Kovatcheva-Datchary *et al*. 2015). A 12-week diet including 20% barley in normal C57BL/6J mice showed that the high ratio of *Actinobacteria*, which is a major acetate

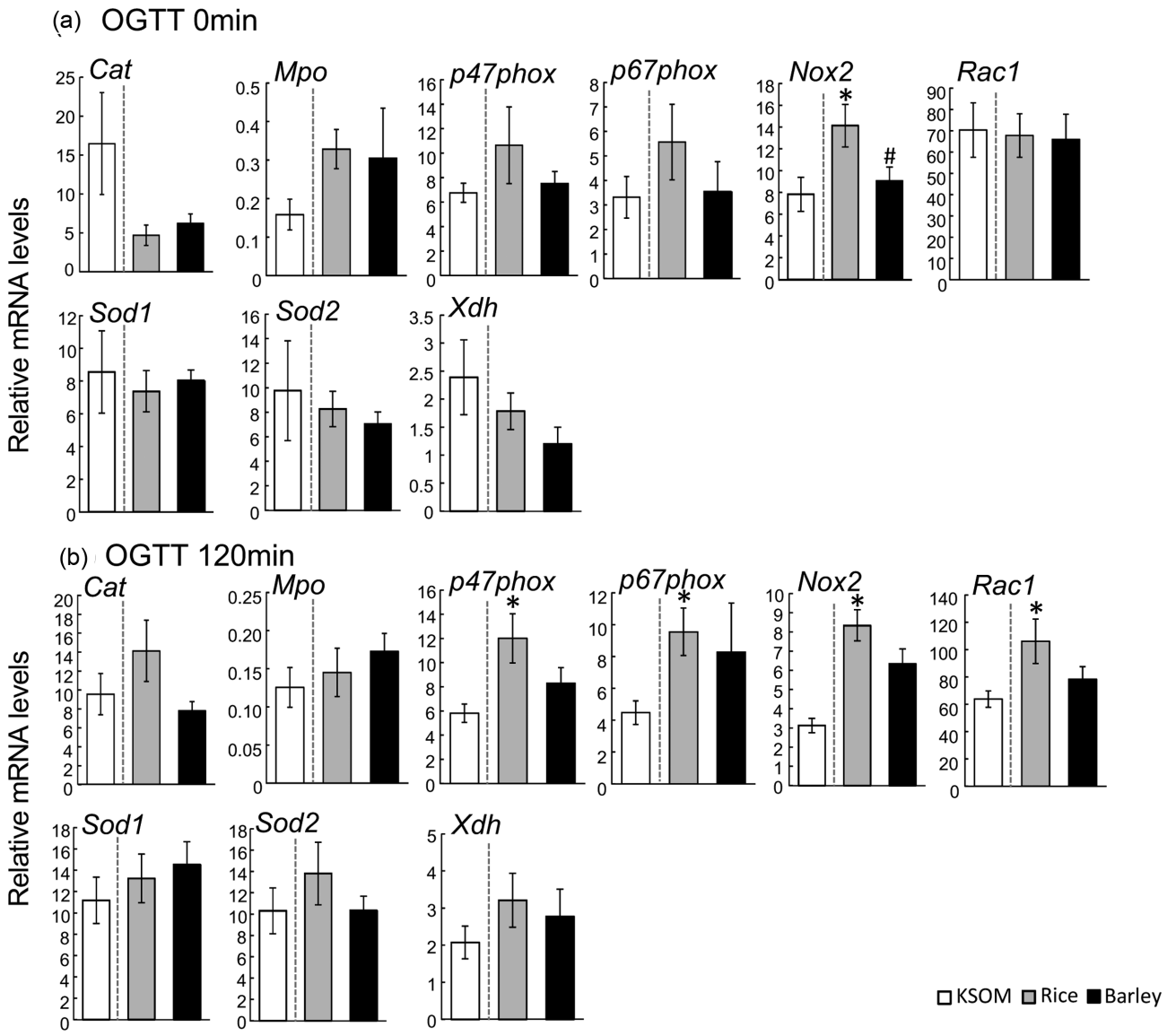


Figure 4. Differences in mRNA expression of oxidative stress-related genes in peripheral leukocytes during OGTT in MEM mice fed a barley diet (M-B; $n = 10$) or a rice diet (M-R; $n = 12$), or KSOM control mice (K-C; $n = 8$) fed a rice diet. Data are expressed as the mean ± SEM for 8-12 animals. Data were analyzed by a Student's *t*-test. Asterisks (*) denote significant differences from K-C mice, where $P < .05$. The pound sign (#) denotes significant differences from M-R mice, where $P < .05$.

producer, and levels of a short-chain fatty acid butyrate, were higher in feces of the barley fed than in control mice (Miyamoto *et al.* 2018). However, our above proposals should be examined in further studies.

There were no differences in the expression of inflammatory genes at fasting, whereas the nonfasting state showed increased expression of several genes in MEM mice fed a rice powder diet compared with KSOM control mice. The nonfasting state is generally considered the postprandial state. In the current study, blood glucose concentrations were higher in the nonfasting than in the fasting period just before the oral glucose loading. Therefore, an explanation for significant differences observed between nonfasting, rather than fasting, MEM mice fed a rice powder diet versus KSOM mice is that the nonfasting period reflects the postprandial state. In addition, although expression levels of many inflammatory genes in peripheral leukocytes were higher, or tended to be higher, in the postprandial period (120 min after oral glucose loading) than the fasting period (0 min), *Tnfa* expression was reduced in the postprandial compared with the fasting period in each group. Previously, we demonstrated that glucose or sucrose loading enhanced *Tnfa* expression in peripheral leukocytes of postprandial and moderately hyperglycemic streptozotocin rats (Tanaka *et al.* 2009) or rats with IGT (Fujimoto *et al.* 2008). Future research should examine whether *Tnfa* expression in peripheral leukocytes is down regulated by oral glucose loading in MEM mice. Additionally, peripheral leukocyte *Nox2* mRNA expression in MEM mice fed a barely diet was lower after oral glucose loading (120 min) and tended to be lower during fasting (for the OGTT) after the 9-week test diet, compared with those fed a rice diet. This effect on *Nox2* expression was not observed in the nonfasting state, and these contrasting findings should be examined in further work.

The nonfasting glucose concentration at 10 weeks after the test diets did not differ between groups, whereas the nonfasting

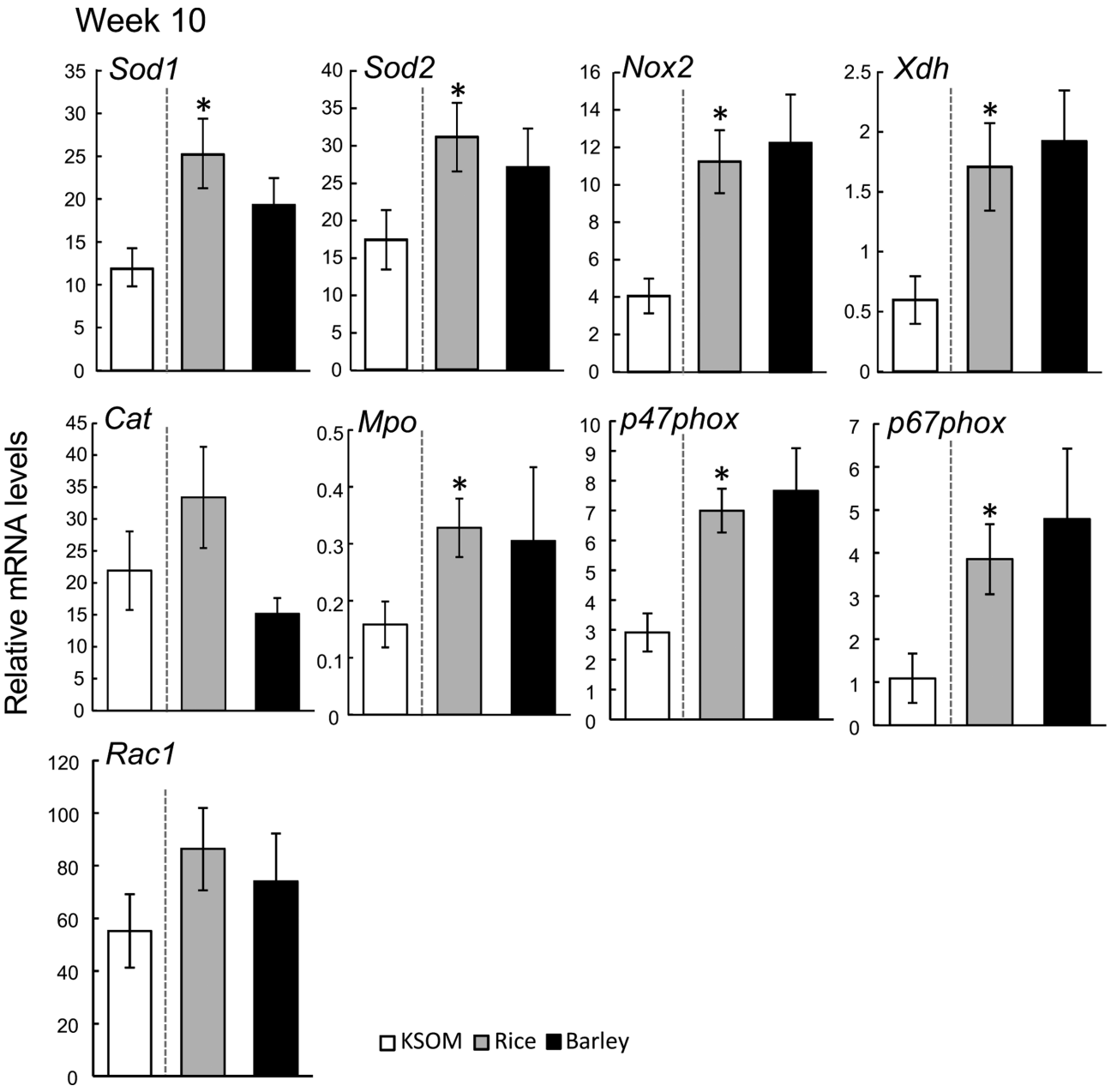


Figure 5. Differences in mRNA expression of oxidative stress-related genes in peripheral leukocytes 10 weeks after administration of test diets. MEM mice were fed either a barley diet (M-B; $n = 10$) or a rice diet (M-R; $n = 12$), and KSOM control mice (K-C; $n = 8$) were fed a rice diet. Data are expressed as the mean ± SEM for 8-12 animals. The data were analyzed by a Student's *t*-test. Asterisks (*) denote significant differences from K-C, where $P < .05$.

insulin concentration tended to be lower in MEM mice fed a barely diet than in those fed a rice diet. It has been reported that barley β-glucan intake suppressed postprandial hyperglycemia and insulin levels in patients with T2DM (Fuse *et al.* 2020). Considering other food factors that can reduce carbohydrate digestion in the small intestine, it has been demonstrated that administration of wheat albumin fractions, which have an inhibitory activity on α-amylase, suppressed postprandial hyperglycemia and insulinemia in mice with IGT (Murayama *et al.* 2009). One mechanism underlying the decreased postprandial elevation of insulin by food factors that reduce carbohydrate digestion and absorption in the small intestine is thought to involve reduction of glucose influx to enterocytes, leading to decreased incretin responses, such as glucose-dependent insulinotropic polypeptide (GIP). Further research should examine whether barely intake reduced GIP release in the small intestine of MEM mice. Oral glucose loading in the OGTT is used to assess pancreatic insulin secretion. Therefore, it is possible that higher insulin secretion in the barley fed MEM mice after oral glucose loading suggests that barely intake may maintain pancreatic insulin secretion, but it was not significant in this study. Therefore, further research should examine whether longer administration or increased barely content, as well as increased animal numbers, leads to significantly increased insulin secretion from the pancreas.

In addition, it remains unclear which components in α-MEM media ultimately affect the diabetic characteristics of adult mice. The α-MEM media has different concentrations of glucose

and protein compared with KSOM media. In addition, future work should examine the specific effects of culturing two-cell embryos with α-MEM media on characteristics of the embryos and offspring.

In summary, we have demonstrated that the MEM mouse model exhibited postprandial hyperglycemia with less obesity, and higher expression of inflammatory genes in peripheral leukocytes compared with KSOM control mice. These results suggest that MEM mice may provide a valuable model for research into nonobese T2DM and the developing risk of atherosclerosis.

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Supplementary material

Supplementary material is available at *Bioscience, Biotechnology, and Biochemistry* online.

Data availability

All data are incorporated into the article and its online supplementary material.

Author contribution

S.K., T.W., and K.M. conceptualized and designed the MEM mice, experiments, and analytical approaches. S.I., M.K., N.U., S.M., A.T., and T.N. carried out the experimental work. M.K., N.U., S.M., A.T., and T.N. contributed to producing MEM mice and sample preparation. S.I. performed the mRNA, Western blotting, and blood chemical analysis and conducted the statistical analyses. S.I. and K.M. wrote the paper with contributions from all authors. T.W., S.K., and K.M. critically revised the manuscript.

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Disclosure statement

No potential conflict of interest was reported by the authors.

References

Adaikalakoteswari A, Balasubramanyam M, Rema M *et al*. Differential gene expression of NADPH oxidase (p22phox) and hemoxygenase-1 in patients with type 2 diabetes and microangiopathy. *Diabet Med* 2006;**23**:666-74.

Boiani M, Gentile L, Gambles VV *et al*. Variable reprogramming of the pluripotent stem cell marker Oct4 in mouse clones: distinct developmental potentials in different culture environments. *Stem Cells* 2005;**23**:1089-104.

Bonora E, Muggeo M. Postprandial blood glucose as a risk factor for cardiovascular disease in Type II diabetes: the epidemiological evidence. *Diabetologia* 2001;**44**:2107-14.

Chen MJ, Norman RK, Heilbronn L. Does in vitro fertilisation increase type 2 diabetes and cardiovascular risk? *Curr Diabetes Rev* 2011;**7**:426-32.

Cohen MM. Perspectives on RAGE signaling and its role in cardiovascular disease. *Am J Med Genet* 2013;**161**:2750-5.

Deodati A, Argemí J, Germani D *et al*. The exposure to uteroplacental insufficiency is associated with activation of unfolded protein response in postnatal life. *PLoS One*. 2018;**13**(6):e0198490.

Domingueti CP, Dusse LMSA, Carvalho MDG *et al*. Diabetes mellitus: the linkage between oxidative stress, inflammation, hypercoagulability and vascular complications. *J Diabetes Complications* 2016;**30**:738-45.

Dubuc PU. The development of obesity, hyperinsulinemia, and hyperglycemia in ob/ob mice. *Metabolism* 1976;**25**:1567-74.

Elnakish MT, Hassanain HH, Janssen PM *et al*. Emerging role of oxidative stress in metabolic syndrome and cardiovascular diseases: important role of Rac/NADPH oxidase. *J Pathol* 2013;**231**:290-300.

Förstermann U. Nitric oxide and oxidative stress in vascular disease. *Pflugers Arch – Eur J Physiol* 2010;**459**:923-39.

Fujimoto S, Mochizuki K, Shimada M *et al*. Variation in gene expression of inflammatory cytokines in leukocyte-derived cells of high-fat-diet-induced insulin-resistant rats. *Biosci Biotechnol Biochem* 2008;2572-9.72

Fukaya N, Mochizuki K, Shimada M *et al*. The α-glucosidase inhibitor miglitol decreases glucose fluctuations and gene expression of inflammatory cytokines induced by hyperglycemia in peripheral leukocytes. *Nutrition* 2009;**25**:657-67.

Fuse Y, Higa M, Miyashita N *et al*. Effect of high β-glucan barley on postprandial blood glucose and insulin levels in type 2 diabetic patients. *Clin Nutr Res* 2020;**9**:43-51.

Galkina E, Ley K. Immune and inflammatory mechanisms of atherosclerosis. *Annu Rev Immunol* 2009;**27**:165-97.

Gao X, Zhang H, Schmidt AM *et al*. AGE/RAGE produces endothelial dysfunction in coronary arterioles in type 2 diabetic mice. *Am J Physiol – Hear Circ Physiol* 2008;**295**(2):H491-8.

Garofano A, Czernichow P, Bréant B. Beta-cell mass and proliferation following late fetal and early postnatal malnutrition in the rat. *Diabetologia* 1998;**41**:1114-20.

Genuth SM, Przybylski RJ, Rosenberg DM. Insulin resistance in genetically obese, hyperglycemic mice. *Endocrinology* 1971;**88**:1230-8.

Jin F, Honma K, Mochizuki K *et al*. Undernutrition in pregnant rats induces glucose intolerance with enhanced expression of inflammation-related genes in peripheral leukocytes of the offspring. *J Nutr Sci Vitaminol* 2019;**65**:534-40.

Kishigami S, Mochizuki K, Wakayama T. The method for producing diabetic animal model and the diabetic animal model. *Japan Patent JP2020-31551A*, 2020 (in Japanese).

Kovatcheva-Datchary P, Nilsson A, Akrami R *et al*. Dietary fiber-induced improvement in glucose metabolism is associated with increased abundance of prevotella. *Cell Metab* 2015;**22**:971-82.

Laakso M. Hyperglycemia and cardiovascular disease in type 2 diabetes. *Diabetes* 1999;**48**:937-42.

Li H, Luo YF, Wang YS *et al*. Using ROS as a second messenger, NADPH oxidase 2 mediates macrophage senescence via

interaction with NF-κB during *Pseudomonas aeruginosa* infection. *Oxidative Med Cell Longevity* 2018;**2018**:9741838.

Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. *Methods* 2001;**25**:402-8.

Lopez-Castejon G, Brough D. Understanding the mechanism of IL-1β secretion. *Cytokine Growth Factor Rev* 2011;**22**:189-95.

Majewska E, Paleolog E, Baj Z *et al*. Role of tyrosine kinase enzymes in TNF-α and IL-1 induced expression of ICAM-1 and VCAM-1 on human umbilical vein endothelial cells. *Scand J Immunol* 1997;**45**:385-92.

Matsuoka T, Tsuchida A, Yamaji A *et al*. Consumption of a meal containing refined barley flour bread is associated with a lower postprandial blood glucose concentration after a second meal compared with one containing refined wheat flour bread in healthy Japanese: a randomized control trial. *Nutrition*. 2020;**72**:110637.

Miyamoto J, Watanabe K, Taira S *et al*. Barley β-glucan improves metabolic condition via short-chain fatty acids produced by gut microbial fermentation in high fat diet fed mice. *PLoS One*. 2018;**13**(4):e0196579.

Murayama Y, Mochizuki K, Shimada M *et al*. Dietary supplementation with α-amylase inhibitor wheat albumin to high-fat diet-induced insulin-resistant rats is associated with increased expression of genes related to fatty acid synthesis in adipose tissue. *J Agric Food Chem* 2009;**57**:9332-8.

Niedowicz DM, Daleke DL. The role of oxidative stress in diabetic complications. *Cell Biochem Biophys* 2005;**43**:289-330.

Osonoi T, Saito M, Mochizuki K *et al*. The α-glucosidase inhibitor miglitol decreases glucose fluctuations and inflammatory cytokine gene expression in peripheral leukocytes of Japanese patients with type 2 diabetes mellitus. *Metabolism* 2010;**59**:1816-22.

Paravicini TM, Touyz RM. NADPH oxidases, reactive oxygen species, and hypertension: clinical implications and therapeutic possibilities. *Diabetes Care* 2008;**31**(Suppl 2):S170-80.

Pober JS. Endothelial activation: intracellular signaling pathways. *Arthritis Res* 2002;**4**:S109-16.

Ridker PM, Everett BM, Thuren T *et al*. Antiinflammatory therapy with canakinumab for atherosclerotic disease. *N Engl J Med* 2017;**377**:1119-31.

Schittenhelm L, Hilkens CM, Morrison VL. β2 integrins as regulators of dendritic cell, monocyte, and macrophage function. *Front Immunol* 2017;**8**:1866.

Scott KA, Yamazaki Y, Yamamoto M *et al*. Glucose parameters are altered in mouse offspring produced by assisted reproductive technologies and somatic cell nuclear transfer. *Biol Reprod* 2010;**83**:220-7.

Tanaka Y, Mochizuki K, Fukaya N *et al*. The α-glucosidase inhibitor miglitol suppresses postprandial hyperglycaemia and interleukin-1β and tumour necrosis factor-α gene expression in rat peripheral leucocytes induced by intermittent sucrose loading. *Br J Nutr* 2009;**102**:221-5.

Tian J, Qiu M, Li Y *et al*. Contribution of birth weight and adult waist circumference to cardiovascular disease risk in a longitudinal study. *Sci Rep* 2017;**7**(1):9768.

Tominaga M, Eguchi H, Manaka H *et al*. Impaired glucose tolerance is a risk factor for cardiovascular disease, but not impaired fasting glucose: The Funagata Diabetes Study. *Diabetes Care* 1999;**22**:920-4.

Wang S, Song R, Wang Z *et al*. S100A8/A9 in inflammation. *Front Immunol* 2018;**9**:1298.

Yan LJ. Pathogenesis of chronic hyperglycemia: from reductive stress to oxidative stress. *J Diabetes Res* 2014;**2014**:137919.

Zhang Y, Ying Y, Zhou L *et al*. Exposure to Chinese famine in early life modifies the association between hyperglycaemia and cardiovascular disease. *Nutr Metab Cardiovasc Dis* 2019;**29**:1230-6.

Zhao H, Song A, Zhang Y *et al*. The association between birth weight and the risk of type 2 diabetes mellitus: a systematic review and meta-analysis. *Endocr J* 2018;**65**:923-33.