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Methylglyoxal reduces molecular responsiveness to 4 weeks of endurance exercise in mouse plantaris muscle

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Short title: methylglyoxal and exercise responsiveness of skeletal muscle

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Abstract

Endurance exercise triggers skeletal muscle adaptations, including enhanced insulin signaling, glucose metabolism, and mitochondrial biogenesis. However, exercise-induced skeletal muscle adaptations may not occur in some cases, a condition known as exercise-resistance. Methylglyoxal (MG) is a highly reactive dicarbonyl metabolite and has detrimental effects on the body such as causing diabetic complications, mitochondrial dysfunction, and inflammation. This study aimed to clarify the effect of methylglyoxal on skeletal muscle molecular adaptations following endurance exercise. Mice were randomly divided into 4 groups (n = 12 per group): sedentary control group, voluntary exercise group, MG-treated group, and MG-treated with voluntary exercise group. Mice in the voluntary exercise group were housed in a cage with a running wheel, while mice in the MG-treated groups received drinking water containing 1% MG. Four weeks of voluntary exercise induced several molecular adaptations in the plantaris muscle, including increased expression of peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC1α), mitochondria complex proteins, toll-like receptor 4 (TLR4), 72-kDa heat shock protein (HSP72), hexokinase II, and glyoxalase 1; this also enhanced insulin-stimulated Akt Ser^{473} phosphorylation and citrate synthase activity. However, these adaptations were suppressed with MG treatment. In the soleus muscle, the exercise-induced increases in the expression of TLR4, HSP72, and advanced glycation end products receptor 1 were inhibited with MG treatment. These findings suggest that MG is a factor that inhibits endurance exercise-induced molecular responses including mitochondrial adaptations, insulin signaling activation, and the upregulation of several proteins related to mitochondrial biogenesis, glucose handling, and glycation in primarily fast-twitch skeletal muscle.

Keywords: exercise-resistance, non-responder, mitochondria, insulin signaling, voluntary wheel running, glycation

New & Noteworthy

This study investigated the effect of methylglyoxal, which is a highly reactive carbonyl metabolite and has detrimental effects on the body, on skeletal muscle adaptations following endurance exercise. Evidences from this study show that methylglyoxal is a factor deteriorating responsiveness to endurance exercise in primarily fast-twitch skeletal muscle. The findings contribute to understand the internal factors that should be focused to maximize the exercise effects.

Introduction

Exercise is a powerful tool for enhancing physical performance and promoting our health. Skeletal muscle adaptations partly provide these benefits after exercise (1). Particularly, enhanced insulin signaling, glucose metabolism, and mitochondrial biogenesis are important molecular adaptations in skeletal muscle in response to endurance exercise training (2, 3). These adaptations are recruited partly through the increased expression of peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC1α) and mitochondria complex proteins (4, 5), insulin signaling activation, and upregulation of glucose handling intermediates, such as glucose transporter (GLUT) 4 and hexokinase (HK) II (6, 7).

However, exercise-induced skeletal muscle adaptations may not occur in some cases, a condition known as exercise-resistance (also called non-responder). Exercise-resistance means that adaptive responses driven by exercise are diminished due to the heterogeneity of factors such as endogenous factors (age, sex, etc.), exogenous factors (exercise intensity, duration, etc.), and molecular responses (proteins, genes, metabolites, etc.) (8). Although it is well established that regular exercise improves whole-body metabolic health in patients with diabetes as indicated in the American Diabetes Association guidelines (9), 7%–63% of them likely have poor exercise responsiveness (10-12). Regarding skeletal muscle responsiveness, De Filippis et al. reported that subjects with insulin-resistance had a reduced response of mitochondrial biogenesis after a single aerobic exercise session (13). Bohm et al. has demonstrated that the failure to improve insulin sensitivity after an 8-week exercise intervention was related to the impaired upregulation of crucial genes for glucose and fatty acid oxidation and mitochondrial oxidative phosphorylation in skeletal muscles (14). Importantly, their study showed that insulin sensitivity before intervention was not different between exercise-resistance and non-exercise-resistance participants (14), indicating the indirect involvement of insulin sensitivity in exercise responsiveness.

Methylglyoxal (MG) is a highly reactive dicarbonyl metabolite, generated mainly during glycolysis, and well-known to contribute to diabetic complications (15, 16). Diabetic patients have 1.5–3 times higher plasma MG levels than healthy people (17). MG induces mitochondrial dysfunction in several tissues (18, 19), generation of advanced glycation end products (AGEs) (20, 21), inflammation through receptor for AGEs (RAGE) (22, 23), and impairing insulin signaling and GLUT4 trafficking in skeletal muscles (24, 25). Our recent study also demonstrated that MG intake for 20 weeks promoted the expression of inflammatory cytokines in the skeletal muscle of mice (26). Given these negative effects of MG on the body, we hypothesized that MG might affect

exercise-associated adaptations. Regarding the relationship between MG and exercise, exercise has been proposed to detoxify MG (27), but no studies have examined the effect of MG on exercise responsiveness.

Therefore, in the present study, we aimed to compare the exercise-associated molecular adaptations of skeletal muscle, including protein expression related to mitochondrial biogenesis and glucose metabolism regulation, between mice undergoing voluntary wheel running with or without MG treatment for 4 weeks. We used lean healthy mice to reduce confounding factors such as obesity- or diabetes-induced humoral changes, insulin resistance, and elevated endogenous MG levels. The exercise period was set to 4 weeks, which is sufficient to reach skeletal muscle adaptations such as enhancing mitochondrial function (28, 29). This model aimed at showing the simple effect of MG on the exercise-associated adaptations of skeletal muscle. This is the first study to show that MG may be a factor that inhibits endurance exercise-induced skeletal muscle molecular responsiveness.

Materials and Methods

Animals and treatment

All animal protocols complied with the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health (Bethesda, MD, USA) and were approved by the Kyoto University Graduate School of Human and Environmental Studies (approval number: 29-A-2).

This study used 48 male C57BL/6NCr mice (9 weeks old) purchased from Shimizu Breeding Laboratories (Kyoto, Japan). After one week of acclimatization, the mice were randomly assigned to 4 groups (n = 12 per group): sedentary control group, voluntary exercise group, MG-treated group, and MG-treated with voluntary exercise group. Mice in the voluntary exercise group were housed in a cage (27×44×19 cm) with a running wheel assembly (16×16×10 cm) purchased from Kyoto L Giken (Kyoto, Japan). The voluntary exercise procedure was carried out as in our previous study (28). Mice in the MG-treated groups received drinking water containing 1% MG. The dose of MG treatment were in accordance with previous research (30, 31). Each group of mice was housed in a standard cage (n = 3 per cage) for 4 weeks under controlled conditions with a 12:12 light-dark cycle, room temperature of 22°C–24°C, and ad libitum access a standard diet and drinking water. The number of mice housed per cage was determined by considering both the accessibility to the running wheel and the cage size. After the 4-week experimental period, the plantaris muscle (PLA), soleus muscle (SOL), and epididymal fat pads were collected with a 72-h resting period after the last bout of exercise. All mice were weighed twice per week.

Insulin stimulation

Muscle incubation was carried out using a modification of previously described methods (32). Immediately after muscle sampling from both legs, the muscles (n = 6 per group) were preincubated in Krebs-Ringer bicarbonate buffer (117 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, and 24.6 mM $NaHCO_3$) containing 2 mM pyruvate, 0.01% bovine serum albumin (Sigma, St. Louis, MO, USA), and 0.005% Antiform SI (FUJIFILM Wako Chemicals, Osaka, Japan) for 10 min. Next, the muscles of the right leg were incubated with 100 μU insulin and the contralateral left muscles without insulin, both for 30 min. The buffers were continuously gassed with 95% O_2–5% CO_2 and maintained at 37°C. The insulin response was expressed as a delta value on the ratio of the responses of the right and left legs in the same mouse to normalize individual differences.

Muscle sample preparation

The muscle samples for western blotting, myosin heavy chain (MyHC) isoform analysis, mitochondrial enzyme activity, and methylglyoxal assay were prepared as described previously (33). Briefly, muscles (n = 6 per group) were homogenized in ice-cold lysis buffer (1:40 wt/vol) containing 20 mM Tris·HCl (pH 7.4), 1% Triton X, 50 mM NaCl, 250 mM sucrose, 50 mM NaF, 5 mM sodium pyrophosphate, 2 mM dithiothreitol, 4 mg/L leupeptin, 50 mg/L trypsin inhibitor, 0.1 mM benzamidine 1 mmol/L Na_3VO_4 and 0.5 mM phenylmethylsulfonyl fluoride, then the homogenates were centrifuged at 16,000 × *g* for 30 min at 4°C, and the supernatants were collected.

Western blotting

The sample proteins (10 μg) were separated on polyacrylamide gels and transferred to polyvinylidene difluoride membranes. Membranes were blocked using EveryBlot blocking buffer (Bio-Rad Laboratories, Hercules, CA, USA) for 5 min, then incubated overnight at 4°C with commercially available antibodies: Akt (9272, Cell Signaling Technology, Danvers, MA, USA), phospho-Akt Ser^{473} (9271, Cell Signaling Technology), aldehyde dehydrogenase 2 (ALDH2) (sc-100496, Santa Cruz Biotechnology, Santa Cruz, CA, USA), glyoxalase 1 (GLO1) (GTX628890, GeneTex, Irvine, CA, USA), GLUT4 (4670-1704, Bio-Rad Laboratories), HKII (2867, Cell Signaling Technology), 72-kDa heat shock protein (HSP72) (ADI-SPA-812, Enzo Life Sciences, New York, NY, USA), AGEs receptor 1 (AGE-R1) (sc-74408, Santa Cruz Biotechnology), OXPHOS Antibody Coktail (ab167109, Abcam, Cambridge, UK), PGC1α (AB3242, Merck Millipore, Burlington, MA, USA), RAGE (sc-365154, Santa Cruz Biotechnology), and toll-like receptor 4 (TLR4) (sc-293072, Santa Cruz Biotechnology). The membranes were then washed with Tris-buffered saline containing 0.1% Tween 20 (TBS-T, pH 7.5) and incubated with anti-mouse IgG (7074, Cell Signaling Technology) or anti-rabbit IgG (7076, Cell Signaling Technology) coupled to horseradish peroxidase for 1 h at room temperature. Each primary and secondary antibody was diluted at 1:10000 with TBS-T. After washing with TBS-T, protein bands were visualized using Chemi-Lumi One Ultra (Nacalai Tesque, Kyoto, Japan) and a bioimaging analyzer (LuminoGraph II, ATTO, Tokyo, Japan). The mean intensity of sedentary control groups in each membrane was serbed as reference to control gel-to-gel variation. Equal protein loading and transfer efficiency was verified by Coomassie Brilliant Blue (CBB) staining of the membranes. The signal intensity of target protein was normalized to total protein (CBB staining intensity). CBB staining intensity did not differ between groups, and its mean coefficient variation (CV) among lanes was 11.9% and 13.9% in PLA and SOL, respectively

(Supplementary Fig. 1). Full blot images and CBB staining images with molecular weight marker band are shown in Supplementary Figs. 2-4.

MyHC isoform analysis

Analysis of MyHC isoform composition (I, IIa, IIx, and IIb) was carried out using a modification of previously described methods (34). The sample proteins (5 μg) were separated on polyacrylamide gels (7%) at 120 V for 19 h in a temperature-controlled chamber at 4°C. After electrophoresis, the gels were incubated with Oriole™ Fluorescent Gel Stain solution (Bio-Rad Laboratories. Hercules, CA, USA) for 1.5 h according to the manufacturer's protocol. After washing with distilled water for 5 min, the gel proteins were visualized by UV light excitation using ImageCapture G3 (Liponics, Tokyo, Japan). MyHC isoform composition in each lane was analyzed using ImageJ software (National Institutes of Health, MD, USA).

Citrate synthase activity assay

The citrate synthase activity was determined from 5% homogenates of skeletal muscle. Analysis was carried out using a modification of previously described methods (35). Briefly, the muscle homogenate was added to the reaction mixture of 70 mM Tris buffer (pH 8.0), 0.1 mM 5,5'-dithiobis (2-nitrobenzoic acid), 0.3 mM acetyl-CoA, and 0.5 mM oxaloacetate. The absorbance of the sample was measured for 5 min at 412 nm at 30°C. Enzyme activity was expressed as micromoles of substrate per minute per gram of muscle weight.

MG assay

The content of MG in muscles was measured using a Methylglyoxal Assay Kit (Colorimetric) (K500-100, BioVision, Milpitas, CA, USA) according to the manufacturer's protocol. MG content in muscles was expressed as nmol/mg protein.

Statistical analysis

Data are presented as means ± SD. The time course changes of body weight and wheel revolution were analyzed using repeated-measured analysis of variance (ANOVA) with exercise and methylglyoxal treatment as between-individual factors. Statistical significance for the other data sets was analyzed using two-way ANOVA with exercise and methylglyoxal treatment as the main factors. Post hoc simple effects tests were conducted via Tukey-Kramer's test. Statistically significance was set at $P < 0.05$. All statistical analyses were performed using BellCurve for Excel software (Social Survey

Research Information, Tokyo, Japan).

Results

MG did not affect body weight, wheel revolution, muscle weight, fat pad weight, and muscle MG level.

Figure 1 shows the mice's physical and behavioral characteristics. The body weight increased gradually in all groups over time (Fig. 1A). Although weight gain was suppressed in the exercise group compared to the sedentary group, MG did not affect this gain (Fig. 1A). MG also did not affect wheel revolution (Fig. 1B). PLA and SOL weight normalized to body weight was increased following exercise, regardless of MG intake (Fig. 1C). Similarly, epididymal fat pad weight normalized to body weight was reduced following exercise, regardless of MG intake (Fig. 1D). The MG level in PLA did not change by MG treatment but decreased following exercise (Fig. 1E). Neither exercise nor MG treatment affected the MG level in SOL (Fig. 1E).

MG suppressed exercise-induced mitochondrial adaptations.

To examine the exercise-induced mitochondrial adaptations in skeletal muscle, we measured the expression of PGC1α and mitochondrial oxidative phosphorylation proteins including NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit8 (complex 1, CI), succinate dehydrogenase [ubiquinone] iron-sulfur subunit (CII), cytochrome b-c1 complex subunit 2 (CIII), mitochondrial cytochrome c oxidase 1 (CIV), and ATP synthase subunit alpha (CV) (Fig. 2).

PGC1α expression in PLA was increased after exercise in the MG-untreated group but not in the MG-treated group (Fig. 2A). Exercise and MG did not affect those in SOL (Fig. 2A). In PLA, the CI, CIII, and CIV expression increased in response to exercise in the MG-untreated group but not in the MG-treated group (Fig. 2B). In contrast, the expression of CII and CV increased regardless of MG treatment (Fig. 3B). In SOL, exercise did not affect the complex proteins, but an MG-induced reduction occurred in CIV (Fig. 2C).

Furthermore, to determine the muscle oxidative capacity, we measured the activity of citrate synthase. The citrate synthase activity in PLA was enhanced following exercise in the MG-untreated group, but not in the MG-treated group (Fig. 3). Exercise did not affect the citrate synthase activity in SOL, but MG treatment decreased the activity (Fig. 3).

MG did not affect MyHC isoform transition following exercise.

To examine the exercise-induced muscle fiber-type adaptation, we measured the expressions of MyHC isoforms in the skeletal muscle (Fig. 4). Exercise increased the

proportion of MyHC type IIa/x and decreased that of type IIb in PLA. It tended to increase the proportion of MyHC type I and decreased that of type IIb in SOL; this indicates that fast-to-slow type fiber transition was induced after exercise. However, MG did not affect this transition.

MG suppressed skeletal muscle insulin signaling activation caused by exercise.

To examine the exercise-induced insulin signaling activation in skeletal muscle, we measured insulin-stimulated Akt Ser^{473} phosphorylation (Fig. 5). Although there was no main effect of exercise and MG treatment on insulin-stimulated Akt Ser^{473} phosphorylation in both muscles, the delta ratio of insulin-stimulated Akt Ser^{473} phosphorylation was tended to increase by exercise in the MG-untreated group, and this response did not occur in the MG-treated group in PLA (Fig. 5A). In SOL, exercise increased the delta ratio of phosphorylation regardless of MG treatment (Fig. 5B).

MG suppressed exercise-induced upregulation of proteins related to mitochondrial biogenesis and glucose metabolism regulation.

To examine the effect of exercise on mediators that regulate mitochondrial biogenesis and glucose metabolism, we measured the expression of TLR4, HSP72, GLUT4, and HKII. In PLA, exercise upregulated the expression of TLR4, HSP72, and HKII in the MG-untreated group, but MG treatment completely or partly attenuated this upregulation (Figs. 6A-D). In SOL, the exercise-induced increase in TLR4 and HSP72 expression was attenuated by MG treatment (Figs. 6A and 6B). On the other hand, exercise increased the expression of GLUT4 regardless of MG treatment in both PLA and SOL (Fig. 6C). Exercise and MG did not affect the expression of HKII in SOL (Fig. 6D).

Exercise and MG affected the expression of enzymes and receptors related to the biological effect of MG.

To examine the effect of exercise and/or MG on the biological effect of MG, we measured the expression of GLO1 and ALDH2 enzymes and AGE-R1 and RAGE receptors. GLO1 and ALDH2 are major enzymes that mediate the detoxification of MG (36). In PLA, GLO1 expression was increased following exercise in the MG-untreated group, but not in the MG-treated group (Fig. 7A), while ALDH2 expression increased regardless of MG treatment (Fig. 7B). In SOL, exercise did not affect GLO1 and ALDH2 expression, but MG treatment reduced the expression of these proteins (Figs. 7A and 7B).

AGE-R1 and RAGE are receptors for AGEs (37). AGE-R1 is a positive regulator of the anti-inflammatory response to AGEs by removing MG-derived AGEs (37), whereas

RAGE recognizes MG-derived AGEs and induces inflammatory responses (38). In PLA, exercise and MG did not affect the expression of AGE-R1 and RAGE (Figs. 7C and 7D). In SOL, exercise increased the expression of AGE-R1 in the MG-untreated group, but not in the MG-treated group (Fig. 7C), whereas MG treatment upregulated RAGE expression (Fig. 7D).

Discussion

We have made several novel findings in the present study regarding the involvement of MG in the exercise-associated molecular adaptations of skeletal muscle. We found MG intake prevented voluntary wheel running exercise-induced mitochondrial adaptations, the activation of insulin signaling (phosphorylation of Akt), and the upregulation of several proteins related to mitochondrial biogenesis (TLR4 and HSP72) and glucose handling (HKII) in primarily fast-twitch PLA muscle. In addition, the enzymes (GLO1 and ALDH2) and receptors (AGE-R1 and RAGE) related to the biological effect of MG were modulated by exercise and/or MG intake.

Mitochondrial adaptation is a well-established molecular change in exercised muscles. Endurance exercise increases the mitochondrial protein abundance and is accompanied by subsequent improvements in muscle oxidative capacity (39, 40). Mitochondrial adaptation following exercise is regulated by several signaling pathways and transcription factors (41). Among these, PGC1α is thought to coordinate exercise-induced mitochondrial biogenesis, including the regulation of mitochondrial DNA transcription and mitochondrial turnover (41, 42). In this study, we found that endurance exercise for 4 weeks upregulated the expression level of PGC1α in PLA; however, this upregulation did not occur during treatment with MG (Fig. 3A); similar changes were seen in the mitochondrial complex proteins (Fig. 3B). Furthermore, the exercise-induced activation of citrate synthase was attenuated with MG treatment (Fig. 4), which is a major regulatory enzyme that controls tricarboxylic acid cycle flux and correlates with skeletal muscle mitochondrial function and oxidative phenotype (43, 44). These findings suggest that MG inhibits the exercise-induced mitochondrial adaptations in fast-twitch skeletal muscle through PGC1α downregulation.

Inflammatory mediators are necessary for exercise-induced mitochondrial adaptation (45, 46). For instance, inflammatory signaling via TLR4, an inflammatory receptor, is also necessary for exercise-induced skeletal muscle metabolic adaptations, including mitochondrial enzyme activation, glucose oxidation, and fatty acid oxidation (46). In addition, HSP72, a stress-inducible form of the HSP70 family, is another factor for mitochondrial biogenesis (47). Several studies indicate that induction of HSP72 following exercise is related to exercise adaptation in the skeletal muscle, although a direct mechanism is still unknown (48). In the present study, the induction of TLR4 and HSP72 following exercise was attenuated with MG treatment (Fig. 7). Previous studies have shown that MG altered the function and stability of HSPs (49, 50). Furthermore, it has also been reported that HSP72/TLR4 pathway was involved in the skeletal muscle responsiveness after exercise via an inflammatory response (51). Therefore, HSP72 and

TLR4, in addition to PGC1α, may affect the MG-induced reduction of exercise-associated mitochondrial adaptations.

Exercise training is a potent intervention for improving insulin action in glucose homeostasis. In contrast, MG is considered a negative regulator for insulin signaling transduction (24, 25). The present study assessed how these conflicting actions affect each other. Both GLUT4 and HKII are determinants of skeletal muscle glucose uptake during exercise (7), and the upregulation of both after endurance exercise training is associated with an increase in insulin-stimulated glucose disposal (6). In addition, mitochondrial dysfunction is also associated with skeletal muscle insulin resistance (52, 53). Several studies have demonstrated an attenuated increase in Akt phosphorylation, HKII expression, PGC1α expression in response to exercise in diabetic individuals (54-56), while the responsiveness of GLUT4 to exercise was preserved (55). In the present study, exercise-induced mitochondrial adaptations were suppressed with MG treatment (Figs. 3 and 4), insulin signal activation (Akt phosphorylation) (Fig. 6), and HKII upregulation (Fig. 7) without affecting GLUT4 response. Taken together, these findings suggest that the failure of skeletal muscle insulin sensitivity after exercise training, as observed in diabetic patients (11, 14), might be due to MG-mediated attenuation of the increases in Akt activation, HKII expression, and mitochondrial function.

The negative effects of MG on skeletal muscle are modulated by several factors, especially the MG detoxification system and receptors of AGEs. The major enzymes which detoxify MG are GLO1 and ALDH2, which eventually metabolize MG to D-lactate and pyruvate (36). In addition, AGEs receptors are also related to the biological effect of MG due to that MG reacts with proteins to produce AGEs (20, 21). To date, more than 10 types of AGEs receptors have been identified (37), with AGE-R1 and RAGE being the most studied receptors. AGE-R1 is a scavenger receptor responsible for the detoxification and clearance of AGEs, and there is an inverse relationship between AGE-R1 expression and AGEs toxicity (37). In contrast, RAGE is a multiligand receptor of the immunoglobulin superfamily. It converts transient cellular stimulation into sustained cellular dysfunction (57). A clinical study has demonstrated that endurance exercise training for 3 months upregulated GLO1 gene expression in the skeletal muscle of elderly men (58). However, there was no evidence regarding the effect of exercise on the protein expression of these enzymes and receptors. The present study is the first to show the adaptive changes in these mediators in response to exercise and MG treatment (Fig. 8). Considering the upregulation of GLO1, AGE-R1, and ALDH2 by exercise, these responses may be involved in some exercise benefits. In fact, it was observed that MG level in PLA was attenuated by exercise (Fig. 2). However, whether these mediators are

involved in exercise adaptations requires further detailed investigation.

The present study showed that exercise-induced adaptations occurred more clearly in PLA compared to SOL. Many studies have reported muscle-specific sensitivity to voluntary exercise training. For instance, our recent studies showed that voluntary exercise for 4 or 8 weeks did not increase mitochondrial proteins in SOL (28, 59). Likewise, other studies demonstrated that exercise training using a running wheel apparatus induced adaptations, including mitochondrial biogenesis, angiogenesis, and aerobic enzyme activations in PLA and the fast-twitch quadriceps muscle, but not in SOL (60-62). Although the mechanisms underlying this phenomenon are unclear, SOL may require a greater aerobic intensity to trigger these adaptations.

Regarding Akt phosphorylation, exercise increased Akt phosphorylation in both PLA and SOL, but MG did not suppress this response in SOL (Fig. 6). Our previous study has recently demonstrated that MG treatment in mice for 20 weeks stimulated MG-derived AGEs accumulation and inflammatory response in fast-twitch extensor digitorum longus muscle but not in slow-twitch SOL (26), indicating that fast-twitch muscle is highly susceptible to MG than slow-type muscle. No other studies have observed muscle-type specific effects of MG. To understand the effect of MG on skeletal muscle from a physiological viewpoint, more detailed studies are needed to clear the muscle-type or muscle fiber-type specificities of MG.

Based on two previous studies (26, 31), the amount of MG intake in the present study is estimated to be 1000-1500 mg/kg body weight/day. A recent study has shown that plasma MG levels did not change in 3-month-old mice that drank 1500 mg/kg body weight/day for 7 days, but urinary levels increased (63), indicating that younger mice have a high ability of the MG clearance system. Because MG is a highly cytotoxic compound and its reactivity is 20,000-fold higher than that of glucose (64), our body tightly controls blood and tissue MG levels through the degradation and excretion, and thereby the MG levels at the blood and tissues are less likely to change. For this reason, it is considered that the skeletal muscle MG levels did not change in the present study (Fig. 2). Previous studies have also demonstrated that the increase in plasma MG level in mice is approximately twofold even after 12-month MG administration (63), and the increase in plasma MG levels in patients with diabetes is 1.5–3 times that in healthy people (31). Taken together, it is suggested that the loading condition of 1% MG for 4 weeks is not at supraphysiological level.

The present study has some limitations. First, we examined molecular adaptations to exercise but did not provide functional performance of mice such as endurance capacity. Additionally, it is necessary to perform not only Akt phosphorylation measurement but

also insulin tolerance test to clarify the effect on insulin sensitivity. Therefore, further in vivo studies are needed to determine whether molecular adaptations are physiologically relevant and induce exercise-resistance in patients with diabetes. Second, to generalize the results obtained in the present study, sex, age, strain of mice, and exercise type should be considered in the model because these factors can affect voluntary wheel running patterns (29). Additionally, sex differences contribute to mitochondrial adaptation by endurance exercise (65). The present study is valuable in showing for the first time the physiological effect of MG on skeletal muscles in young mice, and a more physiological interpretation would be determined by considering the other endogenous and exogenous factors.

In conclusion, the present study results demonstrated that endurance exercise for 4 weeks increased mitochondrial protein expression, stimulated insulin signaling, and increased associated proteins primarily in PLA muscle in the absence of MG treatment. On the other hand, these exercise-induced adaptations were partly attenuated with MG treatment. This suggests that MG is a factor that deteriorates molecular responsiveness to 4 weeks of endurance exercise in primarily fast-twitch skeletal muscle.

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Conflicts of Interest

The authors declare no conflict of interest.

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Supplementary data

The supplementary data are available at Figshare (https://doi.org/10.6084/m9.figshare.16757017).

Figure Legends

Fig. 1

The changes in the body weight (A), cumulative wheel revolutions (B), muscle weight normalized to body weight (C), epidydimal fat pad weight normalized to body weight (D), and muscle methylglyoxal (MG) content (E) following 4-week voluntary exercise and/or MG treatment. Mice were randomly assigned to 4 groups (n = 12 per group): sedentary control group (Sed), voluntary exercise group (Ex), MG-treated group (MG + Sed), and MG-treated with voluntary exercise group (MG + Ex). Mice in the voluntary exercise groups were housed in a cage with a running wheel assembly, while mice in the MG-treated groups received drinking water containing 1% MG. Data are presented as mean ± SD (n = 6-12/group). The bar graph indicates individual data points. The time-course changes of body weight and wheel revolution were analyzed using repeated-measured analysis of variance (ANOVA) with exercise and MG treatment as between-individual factors. Muscle and fat pad weight and MG content data sets were analyzed using two-way ANOVA with exercise and MG treatment as main factors. n.s.: not significant.

Fig. 2

The expression level of peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (PGC1α) (A), and mitochondria complex proteins (CI, CII, CIII, CIV, and CV) in PLA (B) and SOL (C) muscles following 4-week voluntary exercise and/or MG treatment. Data are presented as mean ± SD (n = 6 per group). Individual data points are indicated on the bar graph. Representative immunoblots are shown. Statistical significance was analyzed using two-way ANOVA with exercise and MG treatment as main factors. *: $P < 0.05$ with simple effects tests, n.s.: not significant.

Fig. 3

The citrate synthase (CS) activity in PLA and SOL muscles following 4-week voluntary exercise and/or MG treatment. Data are presented as mean ± SD (n = 6 per group). Individual data points are indicated on the bar graph. Statistical significance was analyzed using two-way ANOVA with exercise and MG treatment as main factors. *: $P < 0.05$ with simple effects tests, n.s.: not significant.

Fig. 4

The changes in the composition of myosin heavy chain (MyHC) isoforms in PLA and SOL muscles following 4-week voluntary exercise and/or MG treatment. MyHC isoforms (type I, IIa/x, and IIb) were determined via sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subsequent fluorescent staining. Data are presented as mean ± SD. Statistical significance was analyzed using two-way ANOVA with exercise and MG treatment as main factors.

Fig. 5

The phosphorylation level of Akt Ser^{473} in PLA (A) and SOL (B) muscles following 4-week voluntary exercise and/or MG treatment. The muscles of the right leg were incubated with 100 μU insulin and the contralateral left muscles without insulin, both for 30 min. The insulin response was expressed as a delta value on the ratio of the responses of the right and left legs in the same mouse. Data are presented as mean ± SD (n = 6 per group). Individual data points are indicated on the bar graph. Representative immunoblots are shown. Statistical significance was analyzed using two-way ANOVA with exercise and MG treatment as main factors. *: $P < 0.05$ on simple effects tests.

Fig. 6

The expression level of toll-like receptor 4 (TLR4) (A), 72-kDa heat shock protein (HSP72) (B), glucose transporter 4 (GLUT4) (C), and hexokinase II (HKII) (D) in PLA and SOL muscles following 4-week voluntary exercise and/or MG treatment. Data are presented as mean ± SD (n = 6 per group). Individual data points are indicated on the bar graph. Representative immunoblots are shown. Statistical significance was analyzed using two-way ANOVA with exercise and MG treatment as main factors. *: $P < 0.05$ on simple effects tests, n.s.: not significant.

Fig. 7

The expression level of glyoxalase 1 (GLO1) (A), aldehyde dehydrogenase 2 (ALDH2) (B), advanced glycation end products-receptor 1 (AGE-R1) (C), and receptor for advanced glycation end products (RAGE) (D) in PLA and SOL muscles following 4-week voluntary exercise and/or MG treatment. Data are presented as mean ± SD. n = 6/group. Individual data points are indicated on the bar graph. Representative immunoblots are shown. Statistical significance was analyzed using two-way ANOVA with exercise and MG treatment as main factors. *: $P < 0.05$ with simple effects tests, n.s.: not significant.

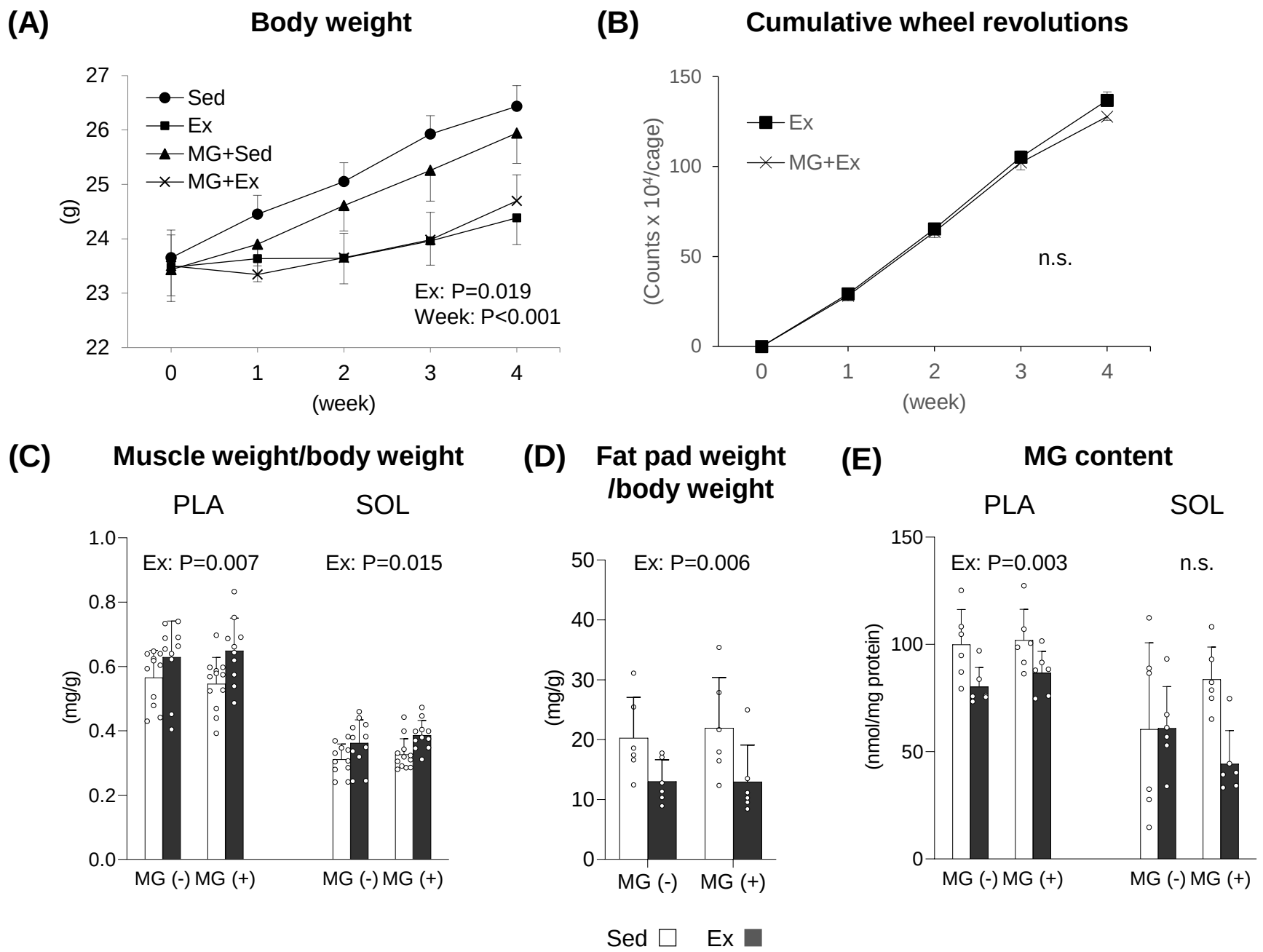
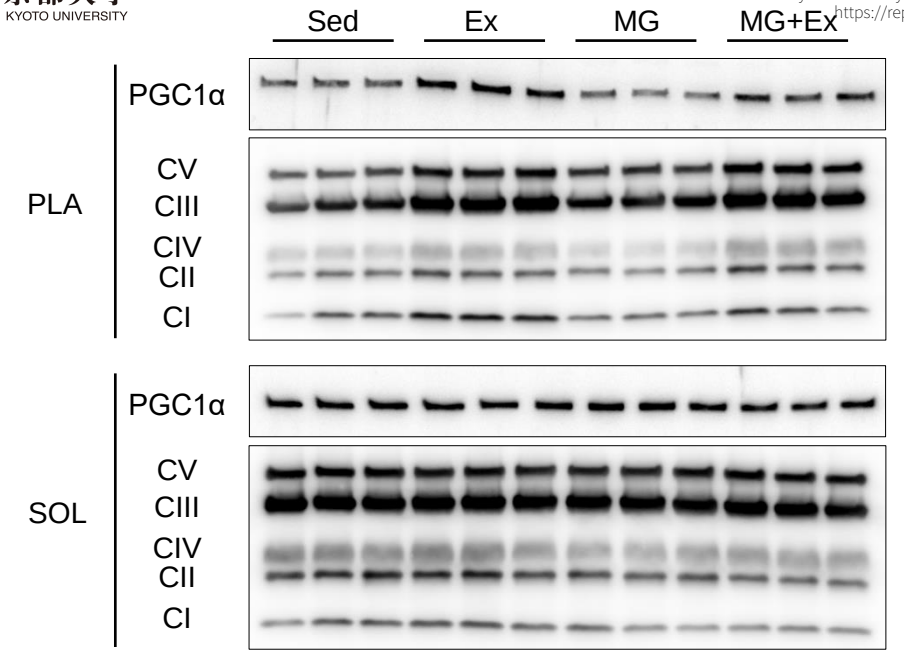
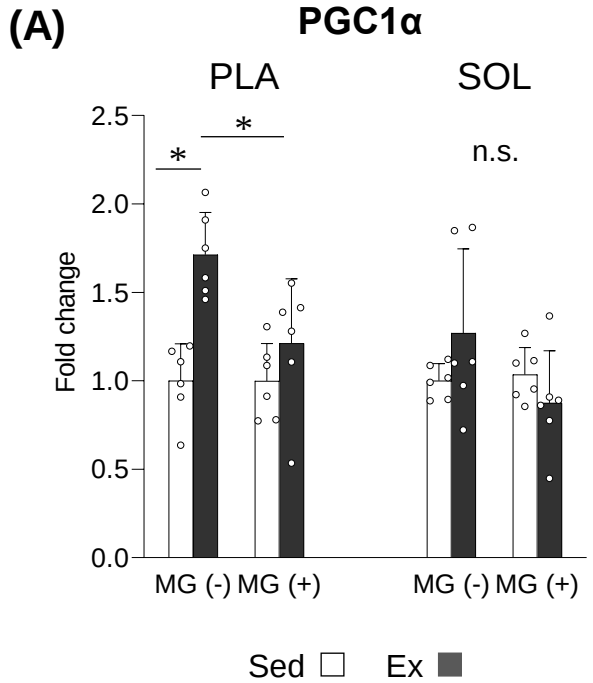
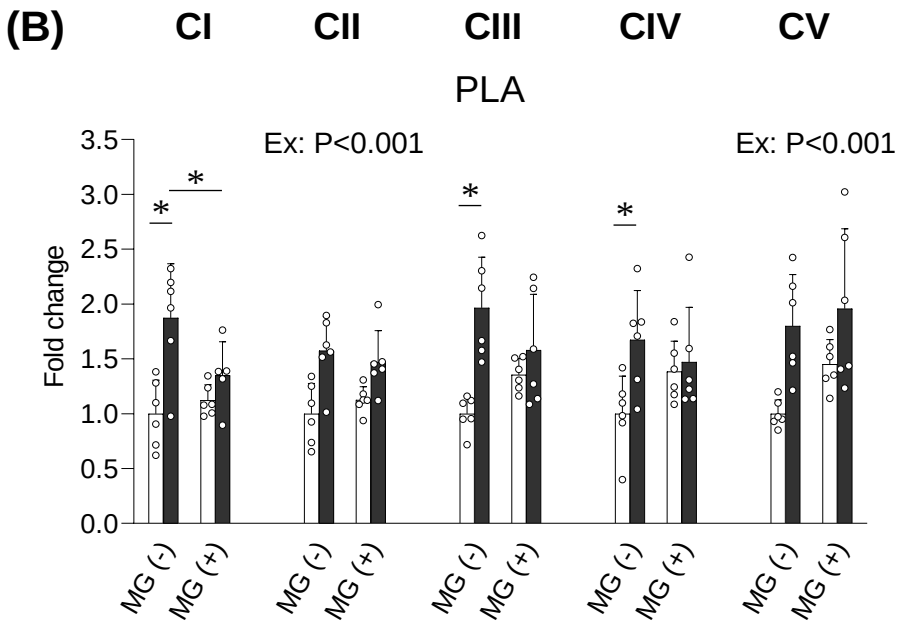


Figure 1







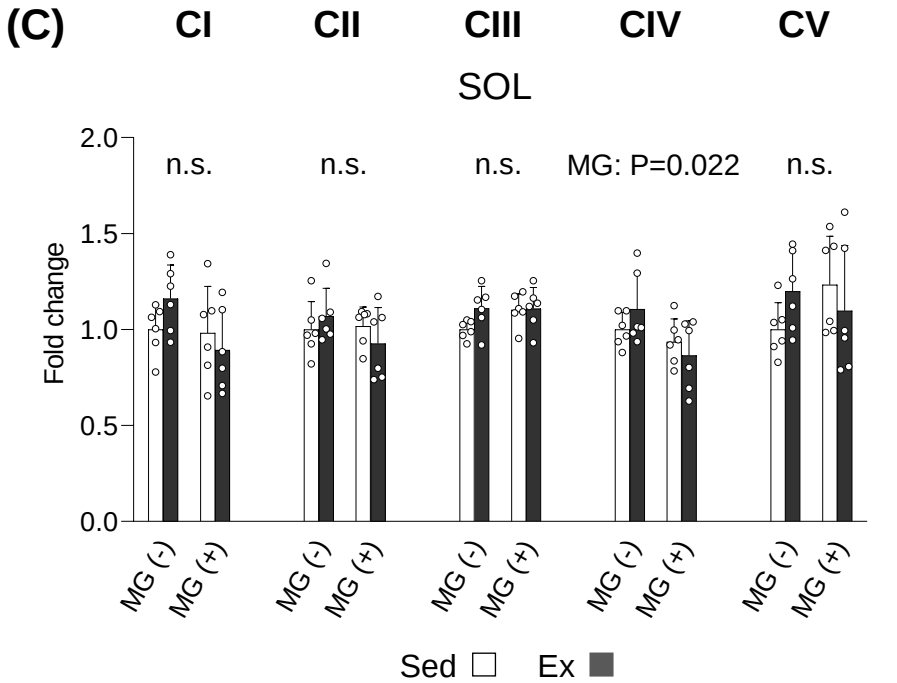
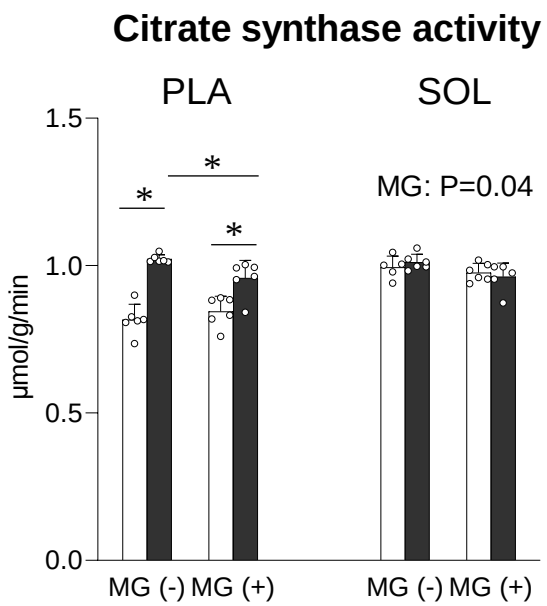


Figure 2



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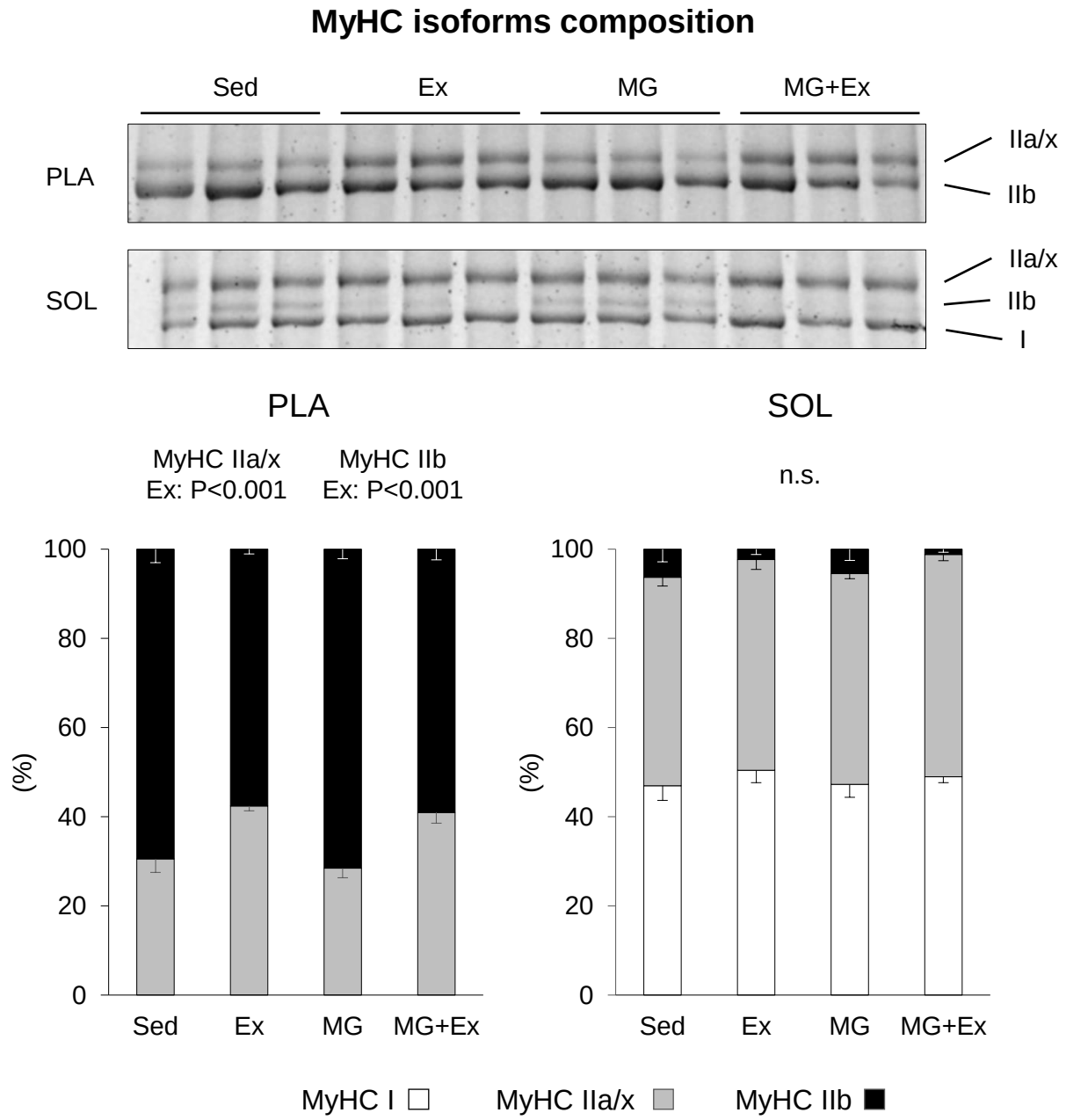

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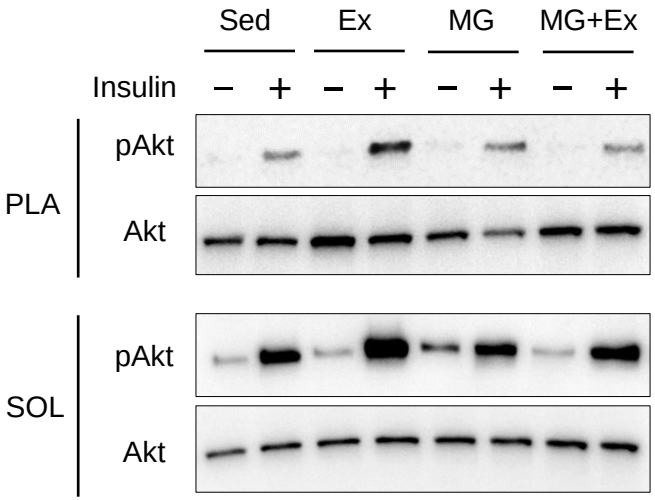


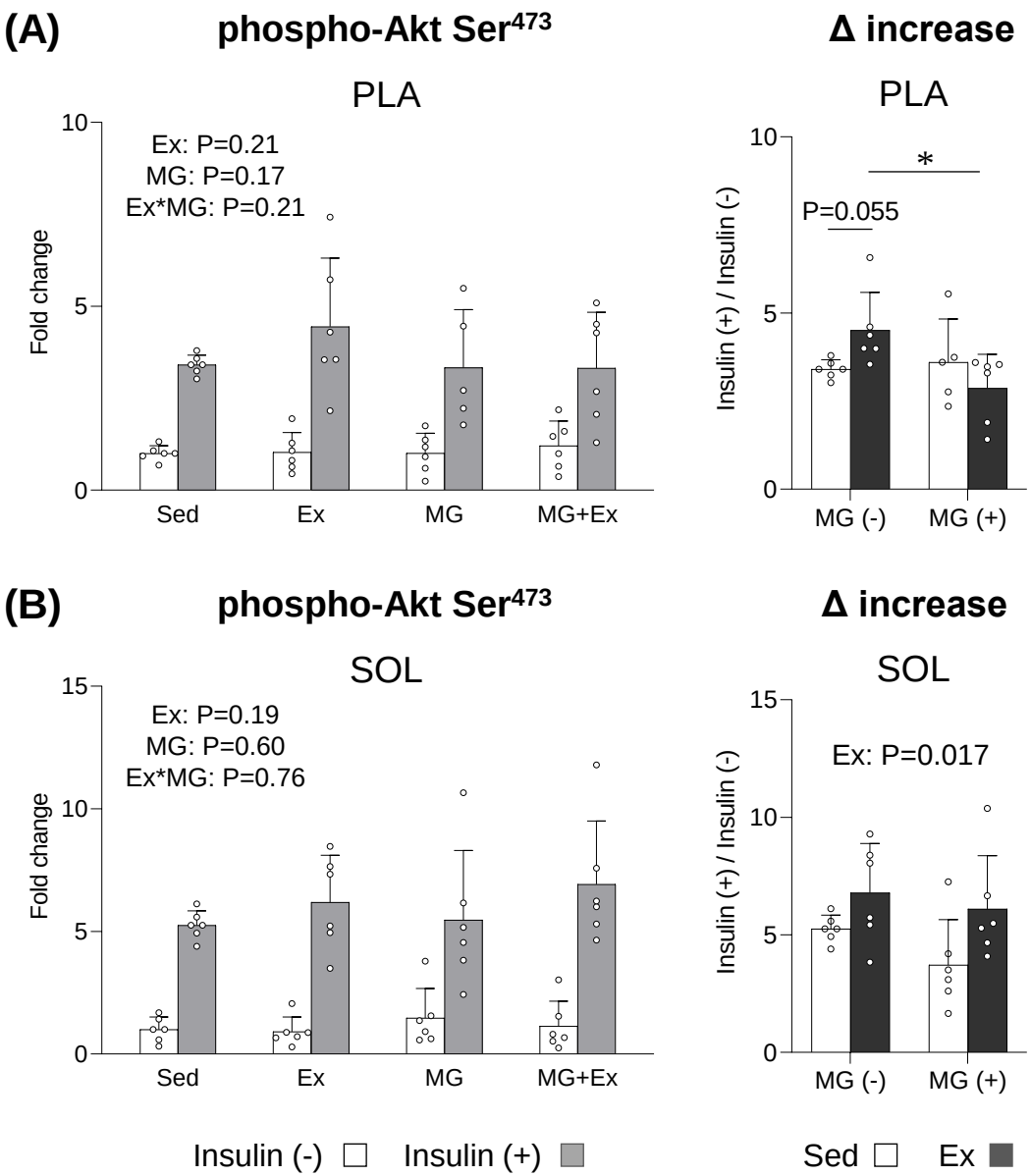
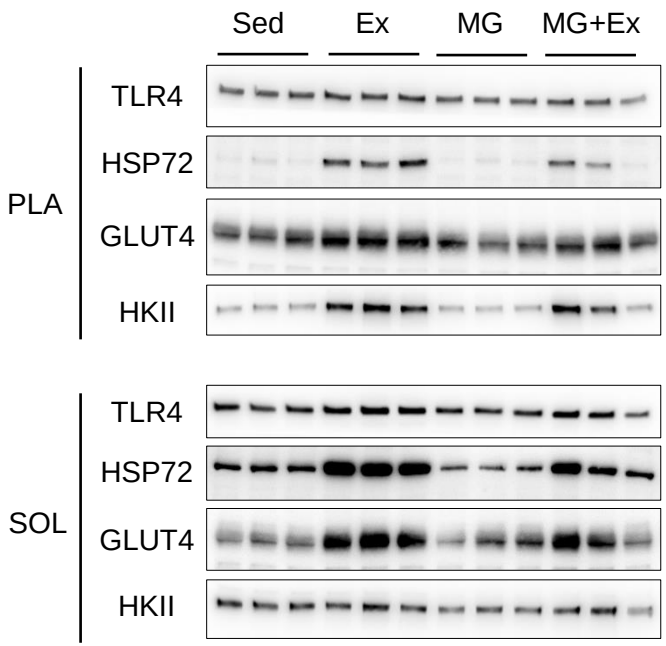


Figure 5



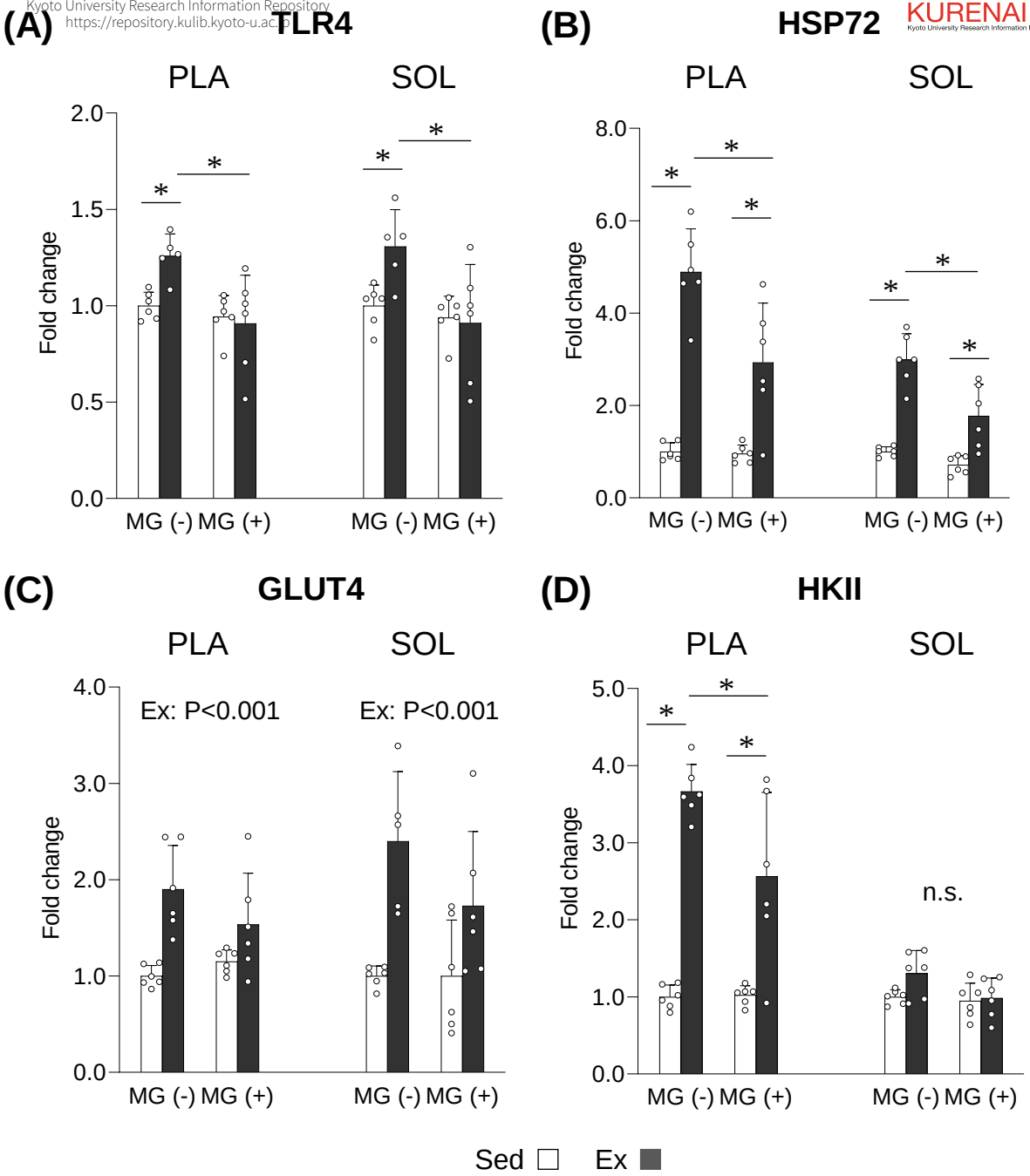
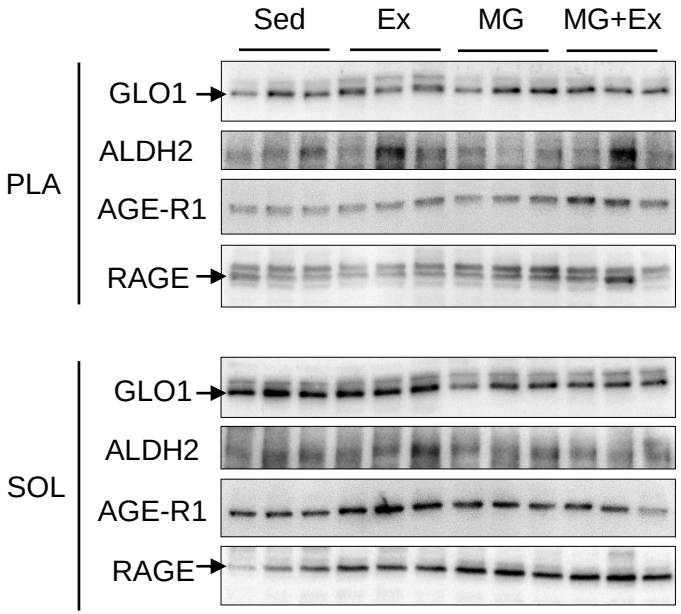


Figure 6



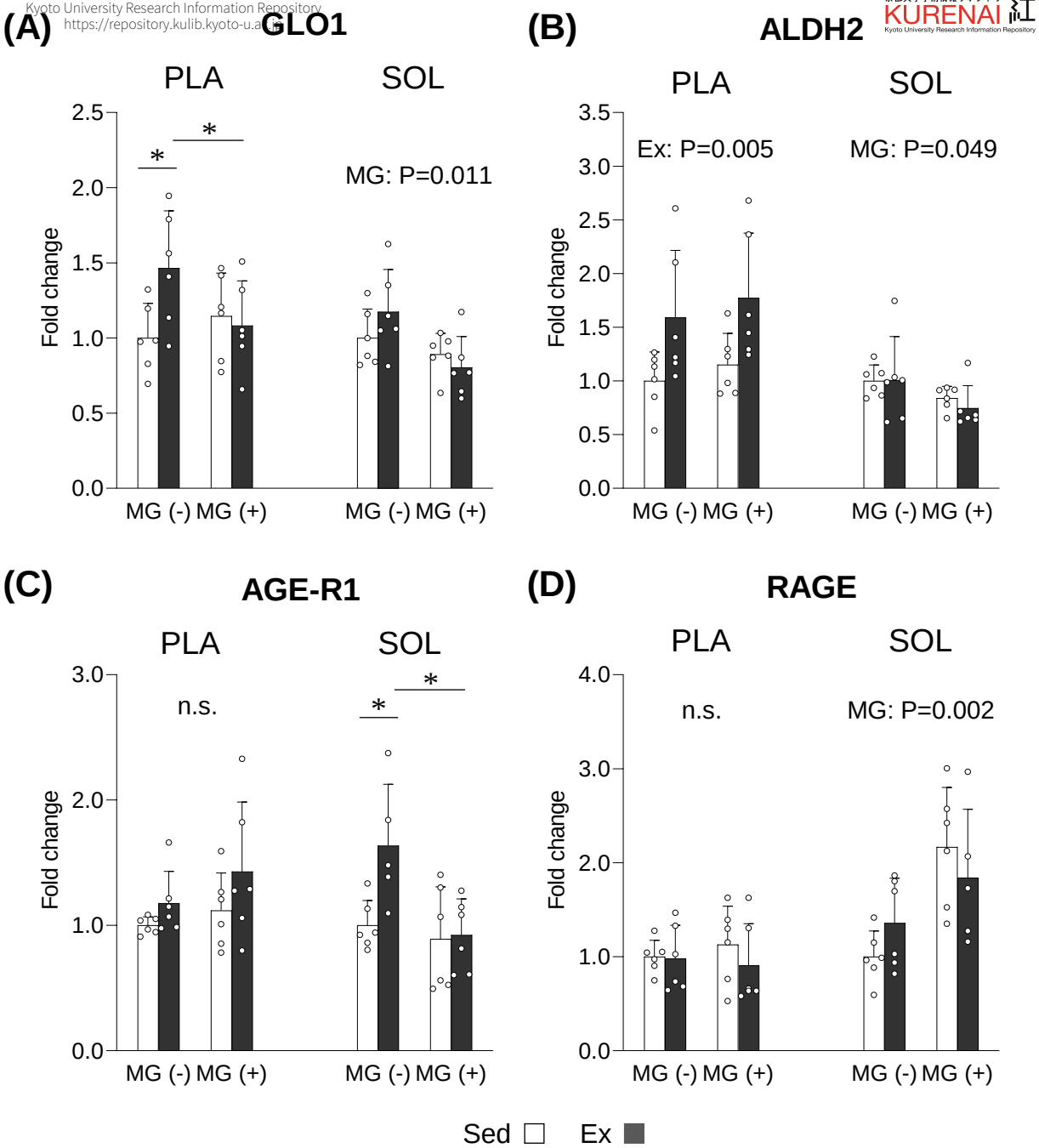


Figure 7