Insulin-like growth factor-I protects against the detrimental effects of advanced glycation end products and high glucose in myoblastic C2C12 cells

Running title: Effects of AGEs and IGF-I on myoblasts

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Abstract

Previous studies suggested that advanced glycation end products (AGEs) and insulin-like growth factor-I (IGF-I) are involved in the mechanism of diabetes-induced sarcopenia. In this study, we examined effects of treatments with AGEs and/or IGF-I for 24 h on myogenic differentiation and apoptosis in mouse myoblastic C2C12 cells. Real-time PCR and Western blot were performed to investigate mRNA and protein expressions, and apoptosis was examined by using a DNA fragment detection ELISA kit. AGE3 significantly decreased mRNA and protein expressions of MyoD and Myogenin, whereas IGF-I significantly increased them and attenuated the effects of AGE3. AGEs significantly decreased endogenous IGF-I mRNA expression and suppressed IGF-I-induced Akt activation. High glucose (22 mM) significantly increased mRNA expression of *Rage*, a receptor for AGEs, while IGF-I significantly decreased it. DNA fragment ELISA showed that AGE2 and AGE3 significantly increased apoptosis of C2C12 cells, whereas IGF-I significantly suppressed the AGE2- and AGE3-induced apoptosis. In contrast, high glucose enhanced AGE3-induced apoptosis. IGF-I significantly attenuated the effects of high glucose plus AGE3 on the mRNA and protein expressions of MyoD and Myogenin as well as the apoptosis. These findings indicate that AGEs inhibit myogenic differentiation and increase apoptosis in C2C12 cells, and that high glucose increases RAGE and enhances the AGE3-induced apoptosis, suggesting that AGEs and high glucose might contribute to the reduction of muscle mass and function. Moreover, IGF-I attenuated the detrimental effects of AGEs and high glucose in myoblastic cells; thus, IGF-I-Akt signal could be a therapeutic target of DM-induced sarcopenia.

Keywords: myoblast, advanced glycation end products, insulin-like growth factor-I, diabetes

mellitus, sarcopenia

Introduction

Sarcopenia is a progressive disease with decreases in skeletal muscle mass and function, resulting in deterioration of activities of daily living and quality of life as well as increases in fall risk and mortality in elderly people [1]. Because the population is worldwide aging, the prevention and treatment of sarcopenia have become an important issue. Accumulating evidence has shown that patients with diabetes mellitus (DM) have an increased risk of sarcopenia [2,3]. Because skeletal muscle is an important tissue involved in insulin action and glucose metabolism [4], it is considered that disturbance of glucose metabolism leads to loss of skeletal muscle and vice versa. Therefore, sarcopenia has recently been recognized as one of the diabetic complications. However, the underlying mechanism of DM-related sarcopenia still remains unclear.

Advanced glycation end products (AGEs) are generated by sequential non-enzymatic chemical glycoxidation of protein amino group [5]. AGEs formation is increased when patients have DM. Several studies have shown that serum AGEs levels are higher in patients with DM than those in healthy subjects [6]. AGEs are known to accumulate in various tissues including eyes, kidney, brain, artery, bone, and muscle [7,8]. Moreover, AGEs have physiological activities and directly impact cell functions through the receptor for AGEs (RAGE) [9]. Therefore, it is suggested that AGEs accumulation in local tissues and increased circulating AGEs may be involved in the mechanism of various diabetic complications [10,11]. Several studies showed that serum AGEs levels are associated with decreased grip power and gait speed in elderly women [12,13]. We recently reported that serum levels of pentosidine, one of the AGEs, are inversely associated with muscle mass index in postmenopausal women with type 2 DM [14]. Moreover, a couple of experimental studies demonstrated that AGEs inhibit myogenic differentiation [15,16].

These finding suggest that AGEs are involved in the pathogenesis of DM-related sarcopenia.

Growth hormone-insulin-like growth factor-I (IGF-I) axis is known to decline with aging; thus, it is thought that the axis is associated with poor physical function or disability in elderly people. Circulating IGF-I, mainly produced in the liver, acts in an endocrine manner and is well-known to exert anabolic effects on muscle mass [17-19]. Because serum IGF-I levels decrease in poorly controlled DM [20,21], low serum IGF-I might be involved in DM-related sarcopenia. We previously showed that serum IGF-I levels were positively and independently associated with muscle mass index in postmenopausal women with type 2 DM [14]. Several studies have shown that IGF-I prevents apoptosis of myoblastic C2C12 cells via activation of Akt [22,23]. Furthermore, Chiu *et al.* recently reported that AGEs induce skeletal muscle atropy and dysfunction through inhibiting Akt signaling pathway in diabetic mice [16]. Moreover, IGF-I stimulated Akt phosphorylation against AGEs and induced myogenic differentiation in C2C12 cells. Taken together, these findings suggest that IGF-I might be a therapeutic target for prevention and treatment of DM-induced sarcopenia.

Previous studies have shown that apoptosis is essential for skeletal muscle development and homeostasis, and that its misregulation has been observed in several myopathies including sarcopenia [24,25]. However, to our knowledge, there are no studies investigating whether AGEs induce apoptosis of myoblasts so far. Moreover, previous studies indicated that high glucose (HG) enhanced the effects of AGEs by increasing RAGE expression in various cells [26-28]; however, it is unknown whether HG affects RAGE expression in myoblasts and whether HG mediates effects of AGEs in the cells. In this study, to determine the potential mechanism by which DM is exacerbating declines in muscle mass and function, we examined whether HG and AGEs increase

apoptosis and inhibit differentiation of C2C12 cells and whether IGF-I treatment inhibits the co-incubation of HG and AGEs-induced detrimental effects.

Materials and methods

Materials

Anti-β-actin antibody and mouse IGF-I were obtained from Sigma-Aldrich (St. Louis, MO, USA). Anti-MyoD and anti-Myogenin antibodies were from Santa Cruz Bioctechnology (Santa Cruz, CA, USA). Anti-total Akt (tAkt), and anti-phospho-Akt (pAkt) antibodies were from Cell Signaling Technology (Beverly, MA, USA). All other chemicals used were of analytical grade.

Cell Culture

Mouse myoblastic C2C12 cells were purchased from the RIKEN Cell Bank (Tsukuba, Japan). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, San Diego, CA, USA) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Invitrogen). The medium was changed twice a week. After the cells reached 80% confluency, the cells were cultured in DMEM with 2% horse serum for 2 days to differentiate into myotube. Then, they were incubated with BSA-free DMEM with vehicle (Cont), 200 μg/mL bovine serum albumin (BSA), 200 μg/mL AGE2 (AGE2), 200 μg/mL AGE3 (AGE3), 100 ng/mL IGF-I (IGF-I), and/or 22 mM glucose (high glucose; HG).

Preparation of AGEs

AGE2, AGE3, and nonglycated BSA were prepared as previously described [15]. AGE2 and AGE3 were prepared by incubating 50 mg/mL BSA (Sigma-Aldrich) with 0.1 M DL-glyceraldehyde (Nacalai Tesque, Kyoto, Japan) and 0.1 M glycolaldehyde (Sigma-Aldrich), respectively, under sterile conditions in 0.2 M phosphate buffer (pH 7.4) containing 5 mM diethylene-triamine-pentaacetic acid at 37 °C or 7 days. Nonglycated BSA was incubated under the same conditions except for the absence of DL-glyceraldehyde or glycolaldehyde as a negative control. Then, low molecular weight reactants and aldehydes were removed using a PD-10 column chromatography and dialysis against phosphate-buffered saline (PBS).

Quantification of gene expression using real-time PCR

For real-time PCR examination, the cells were plated in 6-well plates and cultured as described above. Total RNA was extracted from the cultured C2C12 cells using TRIzol reagent (Invitrogen, San Diego, CA, USA) according to the manufacturer's recommended protocol. We used 2 μg total RNA, which was measured by NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA), for the synthesis of single-stranded cDNA (cDNA synthesis kit; Invitrogen). Then, we used SYBR green chemistry to examine the mRNA expressions of *MyoD*, *Myogenin*, *Igf-I* and *Rage*. A housekeeping gene, *Gapdh,* was used to normalize the differences in the efficiencies of reverse transcription. The primer sequences are listed in Table 1. Real-time PCR was performed with 1 μL of cDNA in a 25 μL reaction volume using the Thermal Cycler Dice Real Time System II (Takara Bio, Shiga, Japan). The double-stranded DNA-specific dye SYBR Green I was incorporated into the PCR buffer provided in the SYBR Green Real-time PCR Master Mix (Toyobo Co. Ltd., Tokyo, Japan) to enable quantitative detection of the PCR

138 product. The PCR conditions were 95 °C for 15 min, followed by 40 cycles of denaturation at
139 94 °C for 15 s, and annealing and extension at 60 °C for 1 min.
140
141 *Western blot analysis*
142 For western blot analysis, the cells were plated in 6-well plates and cultured as described
143 above. After the cells were treated with each agent, they were rinsed with ice-cold PBS and
144 scraped on ice into lysis buffer (Bio-Rad, Hercules, CA, USA) containing 65.8 mM Tris-HCl (pH
145 6.8), 26.3% (w/v) glycerol, 2.1% sodium dodecyl sulphate (SDS), and 0.01% bromophenol blue,
146 to which 2-mercaptoethanol was added to achieve a final concentration of 5%. The cell lysates
147 were sonicated for 20 s. The cell lysates were electrophoresed on 10% SDS-polyacrylamide gels
148 and transferred onto a nitrocellulose membrane (Bio-Rad). The blots were blocked with Tris-
149 buffered saline (TBS) containing 1% Tween 20 (Bio-Rad) and 3% BSA for 1 h at 4 °C. Then, the
150 blots were incubated overnight at 4 °C with gentle shaking with anti-β-actin antibody, anti-MyoD
151 antibody, anti-Myogenin antibody, anti-tAkt antibody, or anti-pAkt antibody as primary
152 antibodies. These blots were extensively washed with TBS containing 1% Tween 20 and were
153 further incubated with a 1:5000 dilution of horseradish peroxidase-coupled IgG of specified
154 animal species (rabbit or mouse) (Sigma-Aldrich) matched to the primary antibodies in TBS for
155 30 min at 4 °C. The blots were then washed, and the signal was visualised using an enhanced
156 chemiluminescence technique. National Institutes of Health (NIH) image software (ImageJ) was
157 used to quantify the signal intensity.
158
159 *Measurement of apoptotic cell death*

C2C12 cells were seeded on 96-well plates at a density of 3,000 cells/well and were incubated overnight in DMEM with 10% FBS and antibiotics. On the next day, the cells were treated with either vehicle, BSA, AGE2, AGE3, HG, and/or IGF-I for 24 h. Then, the cells were lysed, and the supernatant was analyzed in an ELISA for DNA fragments (Cell Death Detection ELISA Plus, Roche Molecular Biochemicals, Indianapolis, IN, USA).

Statistics

All experiments were repeated at least three times. Data are expressed as mean ± standard error of mean (SEM). Statistical analysis for differences among groups was performed by using one-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference test. For all statistical tests, a p value of less than 0.05 was taken to indicate a significant difference.

Results

Effects of AGEs and IGF-I on mRNA expressions of MyoD, Myogenin, and IGF-I in C2C12 cells

We examined the effects of AGEs (200 μg/mL) and IGF-I (100 ng/mL) on the mRNA expressions of *MyoD* and *Myogenin*, both of which were important molecules regulating muscle differentiation, in C2C12 cells by real-time PCR. Treatment with AGE3 for 24 h significantly inhibited the mRNA expressions of *MyoD* and *Myogenin* compared to BSA ($p < 0.01$ and $p < 0.05$, respectively) (Fig. 1A and 1B). IGF-I significantly increased the mRNA expressions of *MyoD* and *Myogenin* compared to Cont (both $p < 0.001$). Moreover, Co-treatment of IGF-I with AGE2 (AGE2+IGF-I) or AGE3 (AGE3+IGF-I) significantly increased the mRNA expressions of

182 *MyoD* and *Myogenin* compared to treatment with AGE2 or AGE3 (at least $p < 0.01$). We then
183 examined the effects of AGEs and IGF-I on the protein expressions of MyoD and Myogenin by
184 Western blot. Treatment with AGE2 or AGE3 for 24 h significantly decreased MyoD and
185 Myogenin protein expressions compared to BSA (at least $p < 0.05$) (Fig. 2A-C), whereas IGF-I
186 significantly increased MyoD and Myogenin protein expressions compared to Cont (both $p <$
187 0.001). Co-treatment of IGF-I with AGE2 (AGE2+IGF-I) or AGE3 (AGE3+IGF-I) significantly
188 increased MyoD and Myogenin protein expressions compared to treatment with AGE2 or AGE3
189 (at least $p < 0.05$).
190
191 *Effects of AGEs and IGF-I on mRNA expression of endogenous IGF-I in C2C12 cells*
192 Real-time PCR showed that AGE2 (200 μg/mL) and AGE3 (200 μg/mL) significantly
193 decreased the mRNA expression of *Igf-I* compared to BSA (both $p < 0.001$). IGF-I (100 ng/mL)
194 significantly decreased the mRNA expression of *Igf-I* compared to Cont ($p < 0.05$), whereas co-
195 treatment of IGF-I with AGE2 (AGE2+IGF-I) or AGE3 (AGE3+IGF-I) significantly increased
196 the mRNA expression of *Igf-I* compared to AGE2 or AGE3 (both $p < 0.001$) (Fig. 1C).
197
198 *Effects of AGEs and IGF-I on Akt activation in C2C12 ells*
199 To examine the effects of AGEs (200 μg/mL) and IGF-I (100 ng/mL) on Akt activation,
200 C2C12 cells were treated with AGEs and/or IGF-I for 24 h, and protein was collected. Western
201 blot showed that IGF-I significantly stimulated pAkt, tAkt, and ratio of pAkt/tAkt compared to
202 Cont (all $p < 0.001$) (Fig. 2D-F). Moreover, AGE2 and AGE3 significantly suppressed tAkt
203 expression compared to BSA ($p < 0.01$ and $p < 0.001$, respectively) (Fig. 2E). IGF-I (BSA+IGF-

204 I) significantly increased pAkt compared to AGE2 or AGE3 ($p < 0.01$ and $p < 0.001$, respectively)
205 (Fig. 2D).
206
207 *Effects of HG, AGE3, and IGF-I on the mRNA expression of RAGE in C2C12 cells*
208 We then examined the effects of HG (22 mM), AGE3 (200 μg/mL), and IGF-I (100
209 ng/mL) on the mRNA expression of *Rage* in C2C12 cells by real-time PCR. HG significantly
210 increased the mRNA expression of *Rage* ($p < 0.001$), while AGE3 tended to decrease it although
211 the difference was not significant (Fig. 3A). In contrast, IGF-I significantly decreased the mRNA
212 expression of *Rage* ($p < 0.001$) (Fig. 3B).
213
214 *Effects of HG, AGE3, and IGF-I on mRNA expressions of MyoD and Myogenin in C2C12 cells*
215 We examined the contribution of HG (22 mM) to the effects of AGE3 (200 μg/mL) on
216 MyoD and Myogenin expressions of C2C12 cells. HG alone did not affect the mRNA expression
217 of *MyoD* or *Myogenin* (Fig. 4A and 4B). AGE3 significantly decreased the mRNA expressions of
218 *MyoD* and *Myogenin* regardless of the presence of HG (all $p < 0.001$). Co-incubation of IGF-I
219 (100 ng/mL) with HG and AGE3 (HG+AGE3+IGF-I) significantly increased the mRNA
220 expressions of *MyoD* and *Myogenin* compared to HG+AGE3 (both $p < 0.001$).
221
222 *Effects of HG, AGEs, and IGF-I on apoptosis of C2C12 cells*
223 The effects of HG (22 mM), AGEs (200 μg/mL), and IGF-I (100 ng/mL) on apoptosis
224 of C2C12 cells were examined by using a DNA fragment detection ELISA kit. Treatment with
225 AGE2 and AGE3 for 24 h significantly increased the apoptosis of C2C12 cells compared to BSA

($p < 0.05$ and $p < 0.01$, respectively) (Fig. 5A). Although HG alone did not affect the apoptosis, co-treatment of HG with AGE3 (HG+AGE3) significantly increased it compared to Cont, HG, and AGE3 (all $p < 0.001$) (Fig. 5B). Co-incubation of IGF-I with AGE2 or AGE3 (AGE2+IGF-I or AGE3+IGF-I) significantly attenuated the apoptotic effects of AGE2 and AGE3 ($p < 0.05$ and $p < 0.01$, respectively) (Fig. 5A). Moreover, co-incubation of IGF-I with HG and AGE3 (HG+AGE3+IGF-I) significantly attenuated the apoptotic affects of HG and AGE3 (HG+AGE3) ($p < 0.05$) (Fig. 5B).

Discussion

The present *in vitro* study showed that AGEs inhibited myogenic differentiation and induced apoptosis in C2C12 cells, and HG increased RAGE mRNA expression and apoptotic effects of AGE3. Moreover, IGF-I suppressed the mRNA expression of RAGE, and attenuated the effects of AGEs on the mRNA and protein expressions of MyoD and Myogenin as well as apoptosis in the presence or absence of HG. These findings suggest that AGEs and hyperglycemia might contribute to the reduction of muscle mass and function, and that IGF-I attenuated the detrimental effects of AGEs and hyperglycemia in myoblastic cells.

We previously showed that AGE2 and AGE3 decreased the differentiation of myoblasts in C2C12 cells [15]. Chiu *et al.* reported that muscle fiber atrophy and accumulation of AGEs in skeletal muscles were markedly increased in patients with DM compared to control subjects, and that AGEs induced muscle atrophy and myogenesis impairment in mouse and human myoblasts via a RAGE-mediated Akt signaling pathway [16]. In this study, AGE2 and AGE3 inhibited the mRNA and protein expression of MyoD and Myogenin as well as Akt protein expression in

C2C12 cells. These findings are consistent with the previous ones [15,16]. Furthermore, the present study firstly showed that AGE2 and AGE3 induced apoptosis of C2C12 cells, and that HG accelerated the detrimental effects of AGEs via increasing RAGE expression. Taken together, these findings suggest that hyperglycemia and high AGEs levels might be involved in the mechanism of DM-related sarcopenia, and that long-term blood glucose control could prevent the risk of sarcopenia in patients with DM.

RAGE expression is essential for AGEs-related diabetic vascular diseases [29]. Previous studies have shown that RAGE is expressed in muscle [30] and plays important roles in skeletal muscle physiology and pathophysiology [31]. Chiu *et al.* showed that AGEs, which were prepared by incubating BSA with D-glucose as AGE1, increased RAGE expression in myocytes [16]; however, AGE2 nor AGE3 affect it in this study. There are dozens of AGEs *in vivo*, the effects may be different among them. For example, Lim *et al*. showed that RAGE is expressed in pancreatic β cells, and that AGE2 and AGE3 increased apoptosis of the cells, compared to AGE1 [32]. Moreover, the effects of AGE3 on myogenic differentiation and apoptosis of C2C12 cells seemed to be stronger than those of AGE2 in this study. Therefore, further studies are necessary to clarify which AGEs are important for development of sarcopenia *in vivo*. In contrast, HG increased RAGE mRNA expression in this study. To our knowledge, this is the first study to show that glucose regulates RAGE expression in myoblastic cells. Thus, these findings suggest that AGEs and hyperglycemia may be involved in muscle mass reduction in aging population, and that AGEs may easily affect muscle especially in patients with DM. However, further *in vivo* studies are necessary to confirm the present findings.

As we expected, IGF-I significantly increased the mRNA and protein expression of

MyoD and Myogenin in C2C12 cells in this study. Moreover, AGE2 and AGE3 significantly inhibited the mRNA expression of endogenous IGF-I and IGF-I-induced phosphorylation of Akt. These findings suggest that AGEs directly decreased myogenesis and indirectly via decreasing IGF-I expression and its signal pathway in microenvironment. Moreover, because IGF-I decreased RAGE mRNA expression, lower IGF-I level may increase RAGE expression. These findings suggest that there might be a vicious cycle between increased AGEs and decreased IGF-I.

Conclusion

In conclusion, AGEs inhibited the expression of MyoD and Myogenin as well as induced apoptosis in C2C12 cells. HG increased RAGE mRNA expression and enhanced the AGEs-induced apoptosis of the cells. These findings suggest that hyperglycemia and AGEs may be involved in the pathophysiology of DM-induced sarcopenia. Moreover, IGF-I decreased RAGE mRNA expression and reversed the detrimental effects of AGEs and HG in myoblastic cells; thus, IGF-I-Akt signal could be a therapeutic target of DM-induced sarcopenia.

Author Contributions

NA and IK designed the study, analysed the data and wrote the manuscript. NA and KT performed the experiments. AT, MN, ST, and TS contributed to design the protocol and discuss the data. TS reviewed and edited the manuscript. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare no conflict of interest.

Abbreviations

AGEs, advanced glycation end products; BSA, bovine serum albumin; Cont, control; DM, diabetes mellitus; FBS, fetal bovine serum; HG, high glucose; IGF-I, insulin-like growth factor-I; RAGE, receptor for AGEs

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398
399

Figure legends

Fig. 1

Effects of AGEs and IGF-I on the mRNA expressions of *MyoD*, *Myogenin*, and *Igf-I* in C2C12 cells

The effects of AGEs (200 μg/mL), IGF-I (100 ng/mL), and AGEs plus IGF-I for 24 h on the mRNA expressions of *MyoD*, *Myogenin*, and *Igf-I* were examined by real-time PCR. (A and B) AGE3 significantly inhibited the mRNA expressions of *MyoD* and *Myogenin* compared to BSA. IGF-I significantly increased the mRNA expressions of *MyoD* and *Myogenin* compared to Cont. Co-treatment of IGF-I with AGE2 or AGE3 (AGE2+IGF-I or AGE3+IGF-I) significantly increased the mRNA expressions of *MyoD* and *Myogenin* compared to treatment with AGE2 or AGE3. (C) IGF-I, AGE2, and AGE3 significantly decreased the mRNA expression of *Igf-I*, whereas the mRNA expression of *Igf-I* was significantly higher in co-treatment of IGF-I with AGE2 or AGE3 (AGE2+IGF-I or AGE3+IGF-I) compared to AGE2 or AGE3. The results are expressed as mean ± SEM ($n \geqq 5$). The housekeeping gene, *Gapdh* was used to normalize the differences in the efficiencies of reverse transcription. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Fig. 2

Effects of AGEs and IGF-I on MyoD and Myogenin expressions as well as Akt activation in C2C12 cells

(A-C) The effects of AGEs (200 μg/mL), IGF-I (100 ng/mL), and AGEs plus IGF-I for 24 h on the protein expressions of MyoD and Myogenin were examined by Western blot. AGE2 or AGE3 significantly decreased MyoD and Myogenin protein, whereas IGF-I and co-treatment of IGF-I

with AGE2 or AGE3 (AGE2+IGF-I or AGE3+IGF-I) increased MyoD and Myogenin protein compared to treatment with AGE2 or AGE3. (A, D-F) The effects of AGEs, IGF-I, and AGEs plus IGF-I for 24 h on Akt activation were examined. IGF-I significantly stimulated pAkt and tAkt expressions and pAkt/tAkt ratio compared to Cont. AGE2 and AGE3 significantly suppressed tAkt expression compared to BSA. IGF-I (BSA+IGF-I) significantly increased pAkt compared to AGE2 or AGE3. The results are representative of at least three different experiments. The results are expressed as mean ± SEM ($n \geqq 3$). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Fig. 3

Effects of HG, AGE3, and IGF-I on the mRNA expression of *Rage* in C2C12 cells

The effects of HG (22 mM), AGE3 (200 μg/mL), and IGF-I (100 ng/mL) for 24 h on the mRNA expressions of *Rage* were examined by real-time PCR. (A) HG significantly increased the mRNA expression of *Rage*, while AGE3 slightly but not significantly decrease it. (B) IGF-I significantly decreased the mRNA expression of *Rage*. The results are expressed as mean ± SEM ($n \geqq 6$). The housekeeping gene, *Gapdh* was used to normalize the differences in the efficiencies of reverse transcription. $***p < 0.001$.

Fig. 4

Effects of HG, AGE3, and IGF-I on the mRNA expressions of *MyoD* and *Myogenin* in C2C12 cells

The effects of HG (22 mM), AGE3 (200 μg/mL) and HG+AGE3 for 24 h on *MyoD* and *Myogenin* mRNA expressions were examined. Also, whether IGF-I (100 ng/mL) attenuates the effects of

444 HG+AGE3 was examined. AGE3 and HG+AGE3 significantly decreased the mRNA expressions
445 of *MyoD* and *Myogenin*. IGF-I significantly increased the mRNA expressions of *MyoD* and
446 *Myogenin* compared to HG+AGE3. The results are expressed as mean ± SEM ($n \geqq 13$). The
447 housekeeping gene, *Gapdh* was used to normalize the differences in the efficiencies of reverse
448 transcription. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.
449
450 **Fig. 5**
451 **Effects of HG, AGEs, and IGF-I on apoptosis of C2C12 cells**
452 (A) The effects of AGEs (200 μg/mL) and IGF-I (100 ng/mL) for 24h on apoptosis of C2C12
453 cells were examined by using a DNA fragment detection ELISA kit. AGE2 and AGE3
454 significantly increased the apoptosis of C2C12 cells. Apoptotic effects of AGE2+IGF-I and
455 AGE3+IGF-I were significantly lower than those of AGE2 and AGE3. (B) The effects of HG (22
456 mM), AGE3, and HG+AGE3 for 24 h on apoptosis of C2C12 cells were examined. Also, whether
457 IGF-I attenuates the apoptotic effects of HG+AGE3 was examined. HG did not affect the
458 apoptosis. AGE3 and HG+AGE3 significantly increased the apoptosis compared to Cont, HG,
459 and AGE3. IGF-I significantly attenuated the apoptotic effects of HG+AGE3. The results are
460 representative of at least three different experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Fig. 1

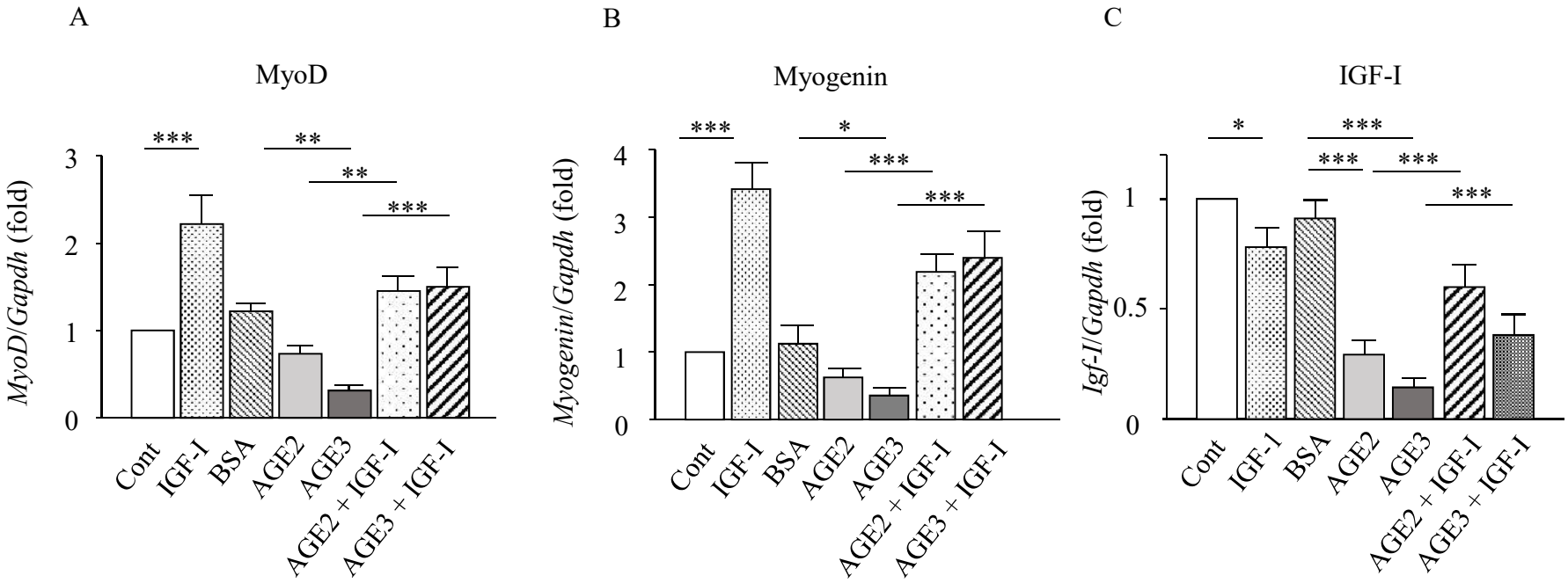
A
MyoD
MyoD/Gapdh (fold)
B
Myogenin
Myogenin/Gapdh (fold)
C
IGF-I
Igf-I/Gapdh (fold)
Cont
IGF-1
BSA
AGE2
AGE3
AGE2 + IGF-1
AGE3 + IGF-1

Fig. 2

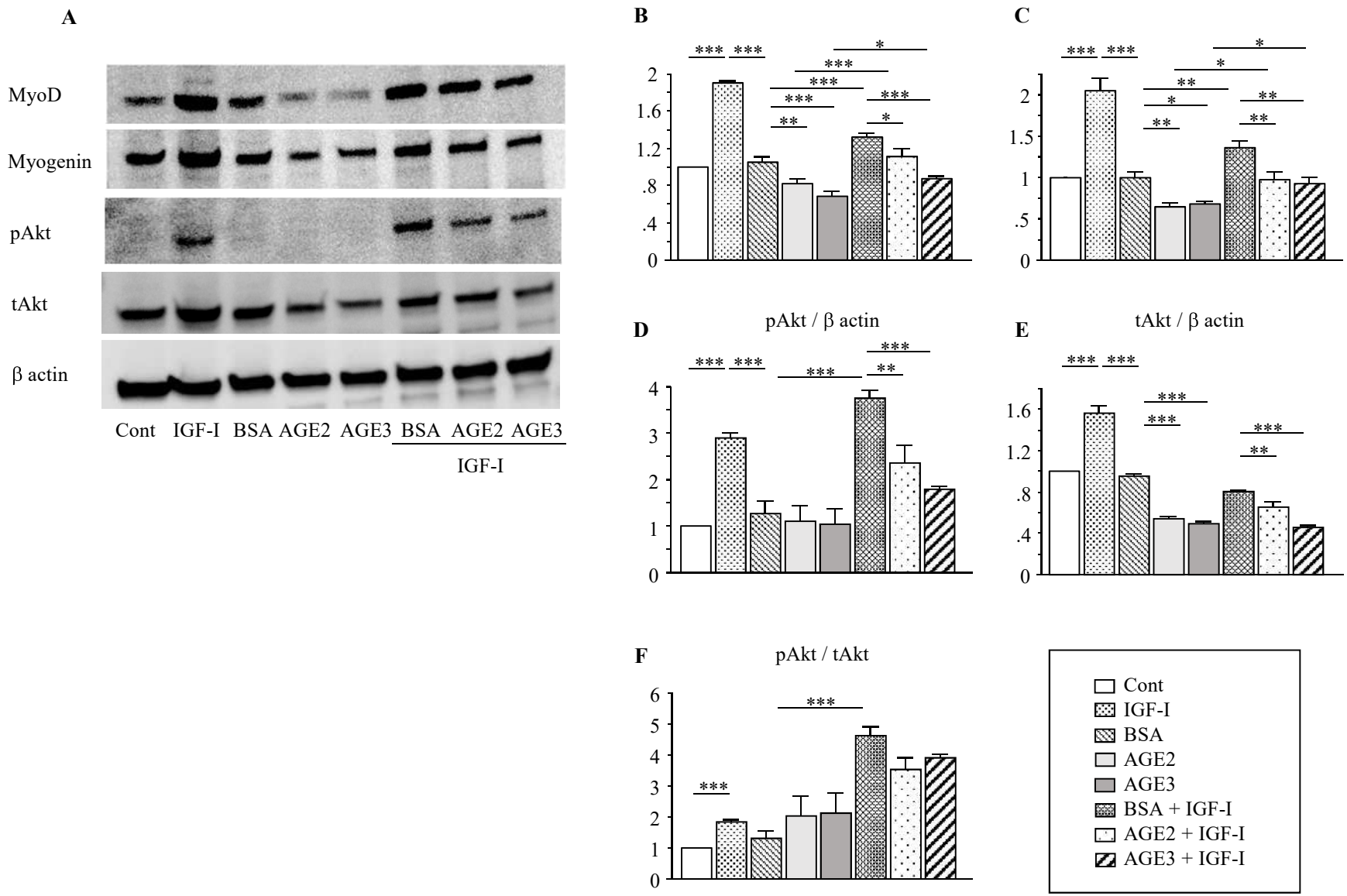
A
MyoD
Myogenin
pAkt
tAkt
β actin
Cont IGF-I BSA AGE2 AGE3 BSA AGE2 AGE3
IGF-I
B
MyoD / β actin
C
Myogenin / β actin
D
pAkt / β actin
E
tAkt / β actin
F
pAkt / tAkt
Cont
IGF-I
BSA
AGE2
AGE3
BSA + IGF-I
AGE2 + IGF-I
AGE3 + IGF-I

Fig. 3

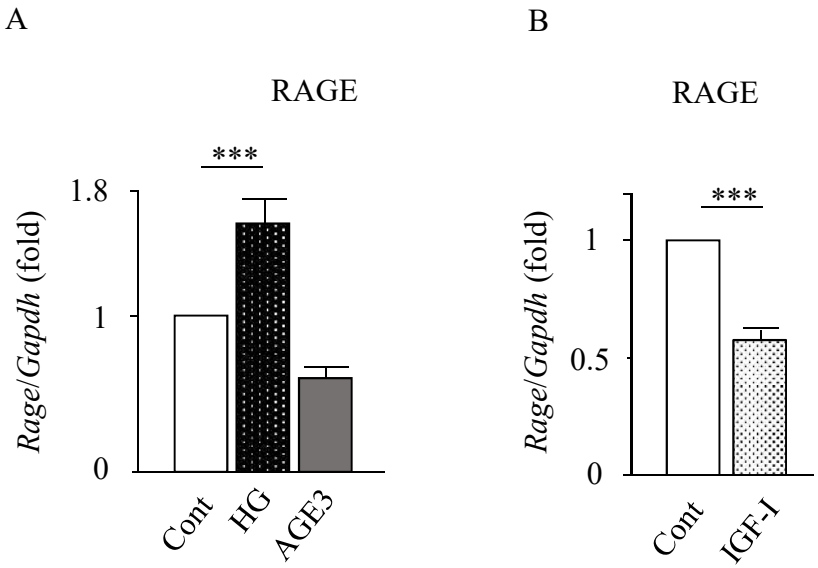
A
RAGE
***
1.8
1
0
Rage/Gapdh (fold)
Cont
HG
AGE3
B
RAGE
***
1
0.5
0
Rage/Gapdh (fold)
Cont
IGF-1

Fig. 4

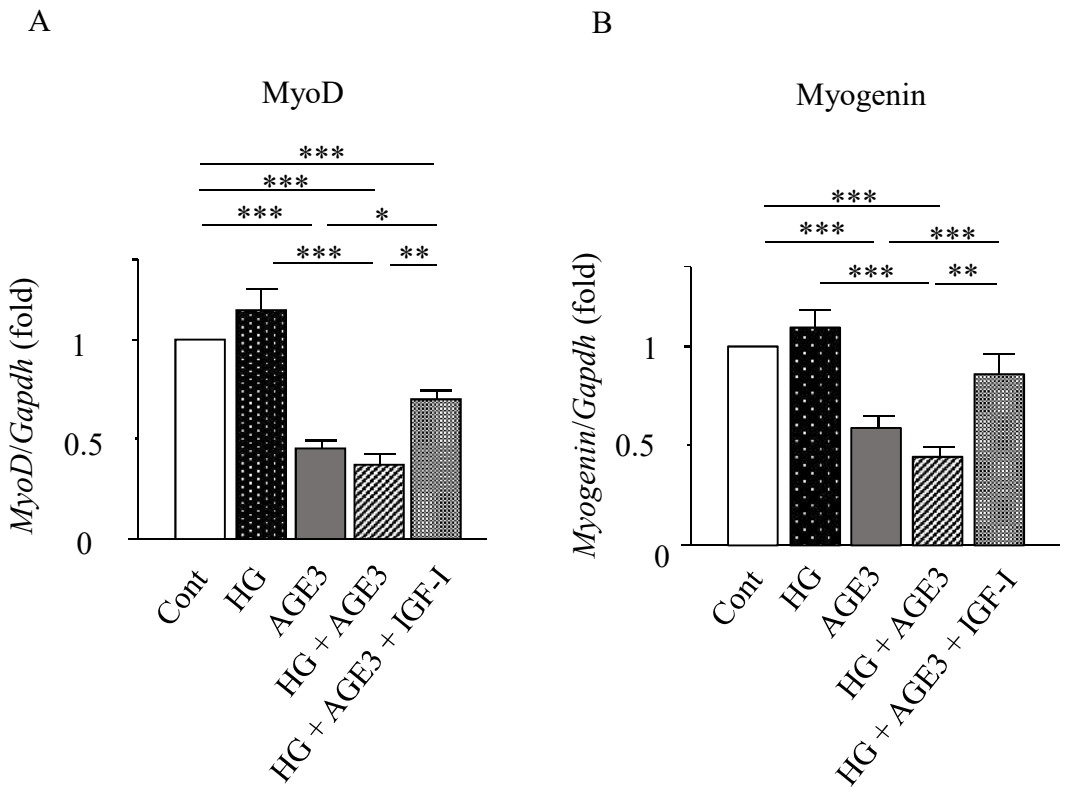
A
MyoD
MyoD/Gapdh (fold)
1
0.5
0
***
***
***
*
***
**
Cont
HG
AGE3
HG + AGE3
HG + AGE3 + IGF-I
B
Myogenin
Myogenin/Gapdh (fold)
1
0.5
0
***
***
***
***
**
Cont
HG
AGE3
HG + AGE3
HG + AGE3 + IGF-I

Fig. 5

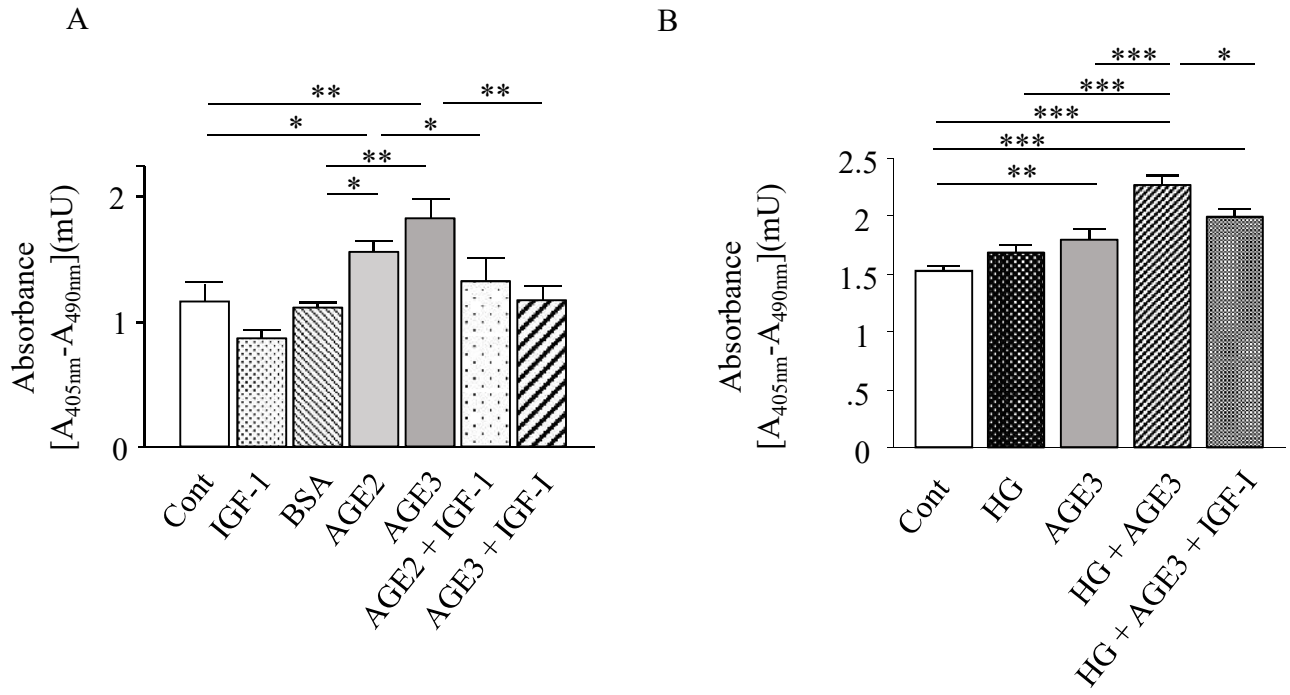
A
B
Absorbance
[A405nm-A490nm](mU)
Cont
IGF-1
BSA
AGE2
AGE3
AGE2 + IGF-1
AGE3 + IGF-1
HG
HG + AGE3
HG + AGE3 + IGF-I

Table 1

Gene name	primers	Accession no.
Gapdh	GTGTACATGGTTCCAGTATGAGTCC AGTGAGTTGTCATATTTCTCGTGGT	GU214026.1
MyoD	GACGGCTCTCTCTGCTCCTT AGTAGAGAAGTGTGCGTGCT	M84918.1
Myogenin	GCTGCCTAAAGTGGAGATCCT GCGCTGTGGGAGTTGCAT	D90156.1
Igf-I	GCTGGTGGATGCTCTTCAGTT TCCGAATGCTGGAGCCATA	NM_001314010.1
Rage	GATTGGAGAGCCACTTGTGCT CCTTCCAAGCTTCAGTTCTTCCT	L33412.1