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The effect of advanced glycation end products on cellular signaling molecules in skeletal muscle

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Abstract The accumulation of advanced glycation end products (AGEs) in the body causes the pathogenesis of aging-related diseases by inhibiting the normal properties and functions of proteins and the modulation of cellular signal transduction. Glycation stress induced by AGEs accumulation has the potential to contribute to sarcopenia: age-related reductions in muscle mass, strength, and function. However, the molecular response to AGEs in skeletal muscle is not fully understood. Therefore, to understand changes in cellular signaling in response to AGEs, this study aimed to investigate the phosphorylation status of phosphoproteins in AGEs-treated skeletal muscle. Treatment of C2C12 skeletal muscle cells with glucose-induced AGEs (0.1 mg/mL) for 5 days suppressed myotube formation, and this was accompanied by Nε-carboxymethyl-lysine accumulation. Reverse phase protein array analysis revealed that treatment with AGEs (glyoxylic-, pyruvate-, glycolaldehyde-, and glucose-induced AGEs) increased phosphorylation at eight phosphorylation sites and decreased phosphorylation at 64 phosphorylation sites. The phosphorylation level of signal transducer and activator of transcription 3 (STAT3) Tyr^{705} was most enhanced, and that of extracellular signal-regulated kinase (ERK) Thr^{202}/Tyr^{204} was most suppressed. Almost all phosphorylation sites related to insulin/insulin-like growth factor 1 signaling were downregulated by AGEs. Increased STAT3 Tyr^{705} phosphorylation and decreased ERK Thr^{202}/Tyr^{204} phosphorylation were observed in the skeletal muscles of mice treated with a diet high in AGEs for 16 weeks. These findings suggest that AGE accumulation impairs cellular signal transduction pathways in skeletal muscle cells, and thereby has the potential to induce skeletal muscle loss.

Keywords : glycation, phosphorylation, signal transducer and activator of transcription 3, extracellular signal-regulated kinase, reverse phase protein array

Introduction

Skeletal muscle is an important tissue of the human body, accounting for approximately 40% of total body mass, and is responsible for voluntary movement control. It is well established that the aging process leads to a decrease in muscle mass and strength. After the age of 50, approximately 2.0% of muscle mass is lost per decade in men[1]. The age-related reductions in muscle mass, strength, and function are known as sarcopenia, and this phenomenon occurs independent of disease[2]. Sarcopenia is associated with multiple adverse outcomes including frailty, disability, morbidity, and mortality[2]. Impaired protein synthesis, accelerated protein degradation, mitochondrial abnormalities, and inflammation are suggested to be potent molecular mechanisms behind sarcopenia[3]. However, knowledge in this area is limited.

Glycation is a biochemical process by which reducing sugars, such as glucose, react and bond non-enzymatically with proteins. Glycation occurs not only in food during the cooking process but also in the human body, which results in the formation of advanced glycation end products (AGEs)[4]. AGE accumulation in the body is closely related to the pathogenesis of aging-related diseases such as diabetes[5,6], Alzheimer's disease[7], cancer[8], and hypertension[9] due to the inhibition of normal properties and

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functions of proteins, and modulation of cellular signal transduction that results from this accumulation. In recent years, glycation stress from endogenous or exogenous AGEs has attracted a lot of attention as it may contribute to sarcopenia. It has been reported that elevated blood Nε-carboxymethyl-lysine (CML), a major immunological epitope among AGEs, is associated with poor grip strength[10] and slow walking speed[11] in elderly people. Furthermore, Japanese men with higher skin AGE levels had lower muscle strength and power[12,13].

AGEs binding to receptors activate a number of downstream signaling pathways and molecules such as mitogen-activated protein kinases, phosphatidylinositol 3-kinase (PI3K), Janus kinase/signal transducers and activators of transcription (STATs), and nuclear factor-kappa B in several cell types[14]. It has been reported that AGEs decrease insulin sensitivity by inhibiting insulin receptor substrate-1/Akt cascade and activating protein kinase Cα in L6 skeletal muscle cells[15,16] and that treating C2C12 skeletal muscle cells with AGEs induces myotube atrophy through activating 5´AMP-activated protein kinase and inhibiting Akt[17]. We have also reported that high AGE intake for 16 weeks inhibited skeletal muscle growth in mice, and this was accompanied by a reduction in the phosphorylation status of 70 kDa ribosomal protein S6 kinase (p70S6K)[18]. These findings indicate that AGE-induced modulation of cellular signaling systems has a crucial influence on skeletal muscle properties, including muscle mass.

To obtain more information about changes in cellular signaling in response to AGEs, this study aimed to investigate the effect of AGEs on the phosphorylation status of phosphoproteins in skeletal muscle by comprehensive proteome analysis. To achieve this goal, we performed reverse phase protein array (RPPA) analysis on C2C12 skeletal muscle cells treated with AGEs. RPPA analysis is an antibody-based protein array that allows for the identification and profiling of target proteins and signaling pathways in a large number of biological samples simultaneously, in a quantitative manner. Using this technology, we found that AGEs impaired cellular signal transduction, inducing skeletal muscle formation.

Material and Methods

AGE treatment in vitro. Cell culture was performed as described previously[19]. In brief, C2C12 cells (DS Pharma Biomedical, Osaka, Japan) were cultured on 6-well culture plates with a type I collagen-coated surface (Biocoat, Becton-Dickinson Labware, Franklin Lakes, NJ, USA). Cells were maintained on growth medium comprising Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Rockville, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum to allow proliferation in a humidified atmosphere with 95% air and 5% CO_2. After the C2C12 myoblasts reached confluence, the culture medium was replaced with a differentiation medium comprising DMEM with 2% heat-inactivated horse serum.

To investigate the effect of AGEs on myogenic differentiation and signaling changes, myoblasts were incubated with or without glucose-induced AGEs (Nippi, Tokyo, Japan) or with BSA (as a control; Millipore, Barrington, MA, USA) at 0.1 mg/mL during the 5 days of differentiation. The medium was replaced with freshly prepared differentiation medium containing AGEs or BSA on day 1 and day 3. For RPPA analysis, on differentiation day 4 C2C12 myotubes were incubated with glyoxylic acid-induced AGEs (AGE-GP01, Cosmo Bio, Tokyo, Japan), pyruvate-induced AGEs (AGE-GP02, Cosmo Bio), glucose-induced AGEs (AGE-GP05, Cosmo Bio), glycolaldehyde-induced AGEs (AGE-GP03, Cosmo Bio), or BSA at a concentration of 0.1 mg/mL for 24 h.

AGE treatment in vivo. Eighteen male ICR mice (5 weeks old) were purchased from Shimizu Breeding Laboratories (Kyoto, Japan). Mice were placed in a room maintained at 22-24°C with a 12:12 h (hour) light/dark cycle and fed a normal diet (AIN-93G, Oriental Koubo, Tokyo, Japan) with water available ad libitum. All animal protocols were carried out in accordance 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: 28-A-2).

AGE administration in vivo was performed as described previously[18]. In brief, mice were randomly assigned to one of two groups; one group was fed a normal diet (AIN-93G unbaked, n = 9), the other a diet high in AGEs (AIN-93G baked for 1 h at 160°C, n = 9), for 16 weeks. The high-AGE diet contained five times as many AGEs compared to the normal diet[20]. At the end of the study period, the extensor digitorum longus muscles were isolated under anesthesia.

Sample preparation and western blot analysis. Sample preparation and western blot analysis were performed as described previously[21,22]. Cells and muscles 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 and 0.5 mM phenylmethylsulfonyl fluoride, and centrifuged at 16,000 g for 30 min at 4°C. The supernatant was collected to determine protein content using the Bradford technique. The protein contents of the supernatant were expressed in mg/mL.

The samples were separated by SDS-PAGE using 10% polyacrylamide gel. After SDS-PAGE, the proteins were transferred to polyvinylidene fluoride membranes (Immobilon-P, Millipore). Next, the membranes were blocked for 1 h using Western BLoT Blocking Buffer (Takara Bio, Otsu, Japan). The membranes were then in-

cubated overnight at 4°C with primary antibodies: AGEs (KH001, TransGenic, Kobe, Japan), extracellular signal-regulated kinase (ERK) Thr^{202}/Tyr^{204} (9101, Cell Signaling Technology, Danvers, MA, USA), ERK (9102, Cell Signaling Technology), myosin heavy chain (MyHC) (14-6503-82, Thermo Scientific, Waltham, MA, USA), STAT3 Tyr^{705} (9131, Cell Signaling Technology), STAT3 (9139, Cell Signaling Technology). The membranes were washed with Tris-buffered saline containing 0.1% Tween 20 (TBS-T, pH 7.5) and reacted with corresponding secondary antibodies for 1 h at room temperature. After a final wash with TBS-T, the protein bands were visualized using ECL select (GE Healthcare, Buckinghamshire, UK) with a bio-imaging analyzer (LuminoGraph II, ATTO, Tokyo, Japan).

RPPA analysis. RPPA analysis was performed by the Carna Biosciences assay service (Kobe, Japan) as described previously[23,24]. In brief, cell lysates were serially diluted (1:1, 1:2, 1:4, and 1:8) and spotted onto glass slides in an array of eight replicates. Signals from slides stained with anti-phospho antibodies were analyzed using the SuperCurve model[25] to obtain a single value of relative concentration for each lysate. After background subtraction, values relative to γ-tubulin were subjected to quantile normalization. Normalized data were used for complete clustering analysis. The log fold change values relative to the BSA control are represented as a heat map.

Statistical analysis. All values were expressed as means ± SE. Groups were compared using the Student's t-test. Values were considered statistically significant at $P < 0.05$. All statistical analyses were performed using Ekuseru-Toukei 2012 software (Social Survey Research Information, Tokyo, Japan).

Results

The effect of AGEs on myotube formation in vitro. To investigate whether AGE treatment affects myotube formation, protein content and MyHC expression were analyzed in myotubes treated with glucose-induced AGEs for 5 days. Both protein content and MyHC expression were lower in AGE-treated myotubes compared to BSA-treated myotubes (Fig. 1A and B). In addition, to identify whether AGE treatment accelerated AGE accumulation, AGE content in myotubes was analyzed by using a monoclonal anti-AGE antibody (6D12) that recognizes CML. AGE (CML)-modified protein content was increased in AGE-treated myotubes (Fig. 1C).

The effect of AGEs on phosphorylation of cellular signaling molecules. To investigate the molecular response to AGEs, the phosphorylation level of cellular signaling molecules was evaluated by RPPA analysis. AGEs are formed not only from glucose, but also from several sugar metabolites[26], so RPPA analysis was applied on myotubes treated with four kinds of AGEs: glyoxylic, pyruvate, glycolaldehyde, and glucose-induced. RPPA analysis applied 180 phosphorylation-site-specific antibodies, and 72 phosphorylation sites were detected in BSA-treated cells. The average level of phosphorylation among the four kinds of AGE treatment increased at eight phosphorylation sites and decreased at 64 (Fig. 2). The five most upregulated phosphorylation sites were STAT3 Tyr^{705}, acetyl-CoA carboxylase Ser^{79}, glycogen synthase kinase (GSK) 3α Ser^{21}, Src Family Tyr^{416} and non-phospho-Src Tyr^{527} (Fig. 3A). The five most downregulated phosphorylation sites were ERK Thr^{202}/Tyr^{204}, small mothers against decapentaplegic (Smad) 2 $Ser^{465/467}$/Smad3 $Ser^{423/425}$, p53 Ser^{37}, eukaryotic translation initiation factor 4G (eIF4G) Ser^{1108}, and the proline-rich Akt substrate of 40 kDa (PRAS40) Thr^{246} (Fig. 3B). Almost all of the phosphorylation sites related to insulin/insulin-like growth factor 1 (IGF-1) signaling were downregulated by AGEs (Fig. 4).

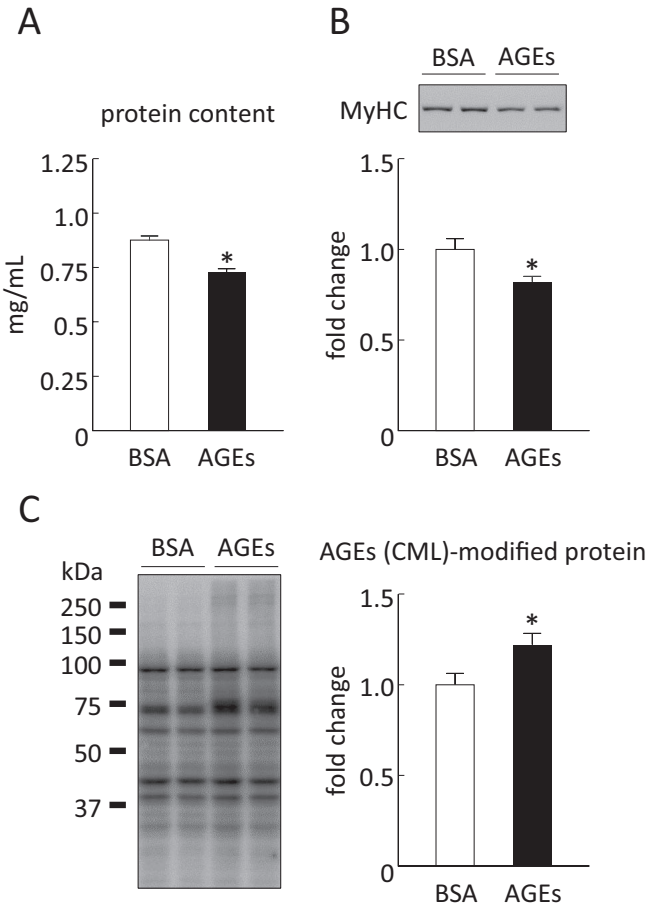

Fig. 1 The effect of AGEs on myotube formation in C2C12 cells.
C2C12 myoblasts were incubated with glucose-induced AGEs or with BSA at 0.1 mg/mL for 5 days of myogenic differentiation. On day 5, protein content (A), myosin heavy chain (MyHC) expression (B), and AGE (Nε-carboxymethyl-lysine (CML)) content (C) were determined. Representative immunoblots are shown. Data are expressed as mean ± SE, n = 6/group. *P < 0.05 vs. BSA.

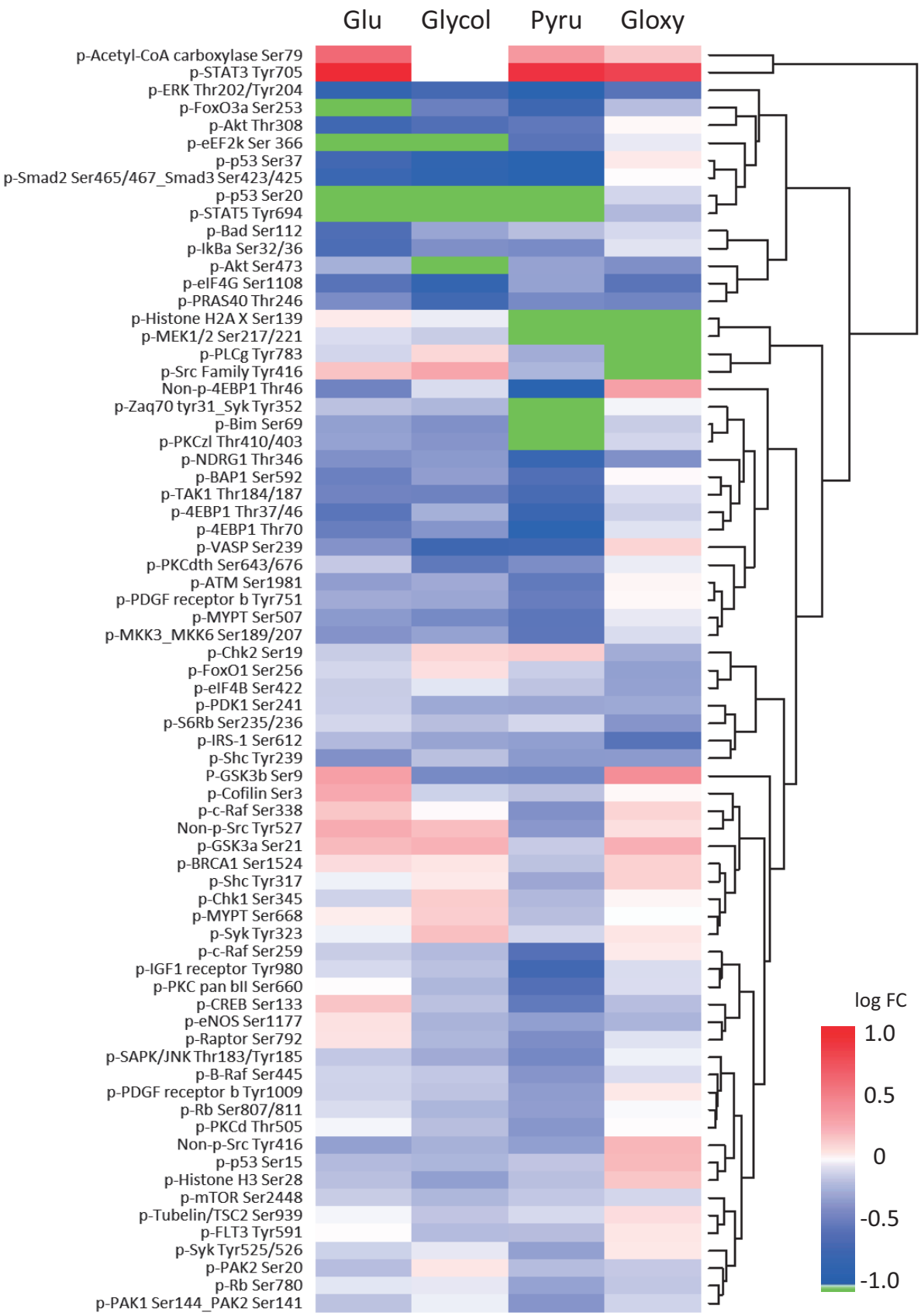

Fig. 2 Phosphoprofiling of signaling molecules by RPPA analysis.
C2C12 myotubes on day 4 of myogenic differentiation were incubated with glyoxylic acid-induced AGEs (Glyoxy), pyruvate-induced AGEs (Pyru), glycolaldehyde-induced AGEs (Glycol), glucose-induced AGEs (Glu), or BSA at 0.1 mg/mL for 24 h. After that, reverse phase protein array (RPPA) analysis was carried out. RPPA data were used for complete clustering analysis. The relative log fold-change (FC) values of 72 phosphorylation sites are represented as a heat map. Red, increased; Blue, decreased; Green, below the quantitation limit.

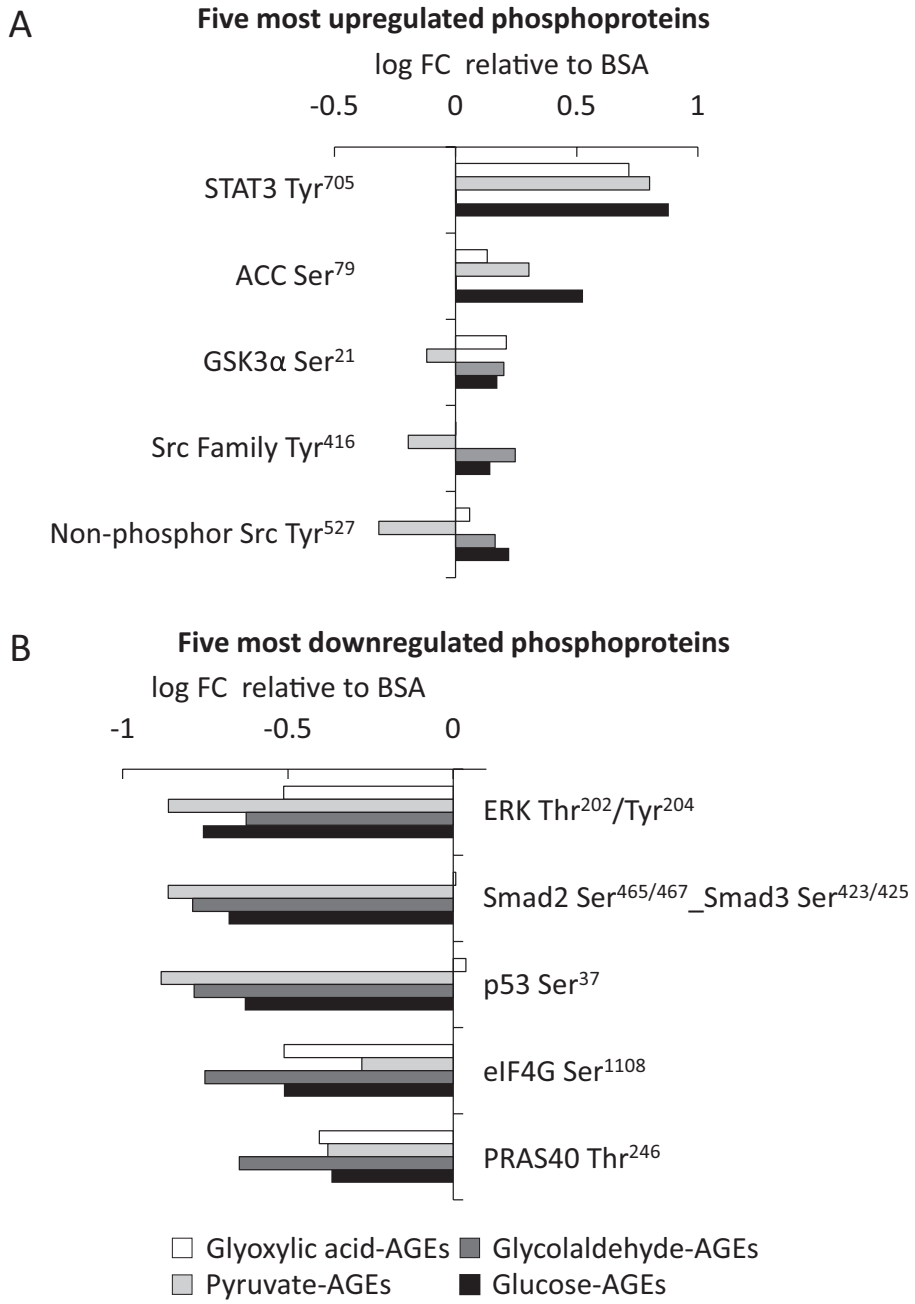

Fig. 3 Five most upregulated and downregulated phosphoproteins by AGEs.
C2C12 myotubes on day 4 of myogenic differentiation were incubated with glyoxylic acid-induced AGEs, pyruvate-induced AGEs, glycolaldehyde-induced AGEs, glucose-induced AGEs, or BSA at 0.1 mg/mL for 24 h. After that, RPPA analysis was performed. The five most upregulated and downregulated phosphoproteins are represented as the relative log fold-change (FC) values. STAT, signal transducer and activator of transcription; ACC, acetyl-CoA carboxylase; GSK, glycogen synthase kinase; ERK, extracellular signal-regulated kinase; Smad, small mothers against decapentaplegic; eIF4G, eukaryotic translation initiation factor 4G; PRAS40, proline-rich Akt substrate of 40 kDa.

The effect of AGEs on skeletal muscle STAT3 and ERK phosphorylation in vivo. To investigate whether these signaling changes are also observed in skeletal muscle in vivo, the phosphorylation level of STAT3 Tyr^{705} and ERK Thr^{202}/Tyr^{204}, which were the molecules most affected by AGE treatment in vitro (Fig. 3), was investigated in the skeletal muscle of mice treated with a diet high in AGEs for 16 weeks. We have previously shown that this treatment induces skeletal muscle loss[18]. The phosphorylation of STAT3 Tyr^{705} was higher and that of ERK Thr^{202}/Tyr^{204} was lower in the high-AGE diet group compared to the normal diet group (Fig. 5A and B). There were no changes in the total expression level of STAT3 and ERK (Fig. 5A and B).

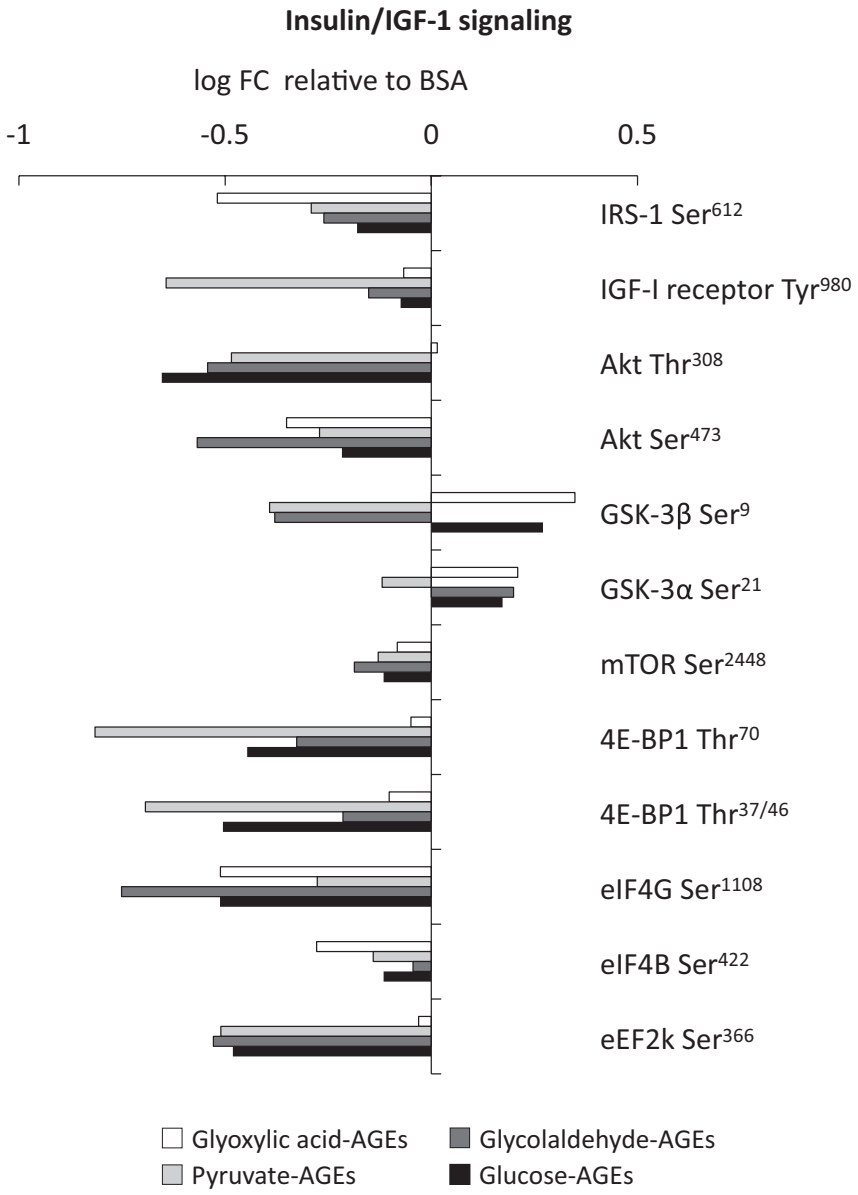

Fig. 4 The phosphoproteins related to insulin/IGF-1 signaling.
C2C12 myotubes on day four of differentiation were incubated with glyoxylic acid-induced AGEs, pyruvate-induced AGEs, glycolaldehyde-induced AGEs, glucose-induced AGEs, or BSA at 0.1 mg/mL for 24 h. After that, RPPA analysis was performed. The phosphoproteins related to insulin/IGF-1 signaling are represented as the relative log fold-change (FC) values. IRS-1, insulin receptor substrate-1; IGF-1, insulin-like growth factor-1; GSK, glycogen synthase kinase; mTOR, mammalian target of rapamycin; 4E-BP1, eukaryotic translation initiation factor 4E binding protein 1; eIF, eukaryotic translation initiation factor; eEF2k, eukaryotic elongation factor 2 kinase.

Discussion

It has been reported that AGEs accumulate with aging in human blood and tissues such as the lens, skin, aorta, liver, and skeletal muscle[27]. Animal experiments have revealed the intracellular accumulation of AGEs in the fast- and slow-twitch muscles of older rats (>27 months)[28,29]. In addition, elevated AGEs in blood or skin are negatively correlated with muscle mass, grip strength, and walking speed in elderly people[10-13]. These observations suggest that AGE accumulation in the body is a cause of sarcopenia. In the present study, we found that treatment with glucose-induced AGEs for 5 days suppressed myotube formation in C2C12 skeletal muscle cells, and that this was accompanied by AGE accumulation (Fig. 1). This finding corresponds with that of a previous study, which demonstrated that 1 day of glucose-induced AGE treatment reduced the myotube diameter in C2C12 skeletal muscle cells[17]. Furthermore, the AGE-induced reduction of the myotube diameter was attenuated by co-treatment with alagebrium chloride, which degrades AGEs[17]. This evidence suggests that AGEs may reduce myogenic capacity and thereby induce skeletal muscle loss.

AGE (CML)-modified proteins were observed especially

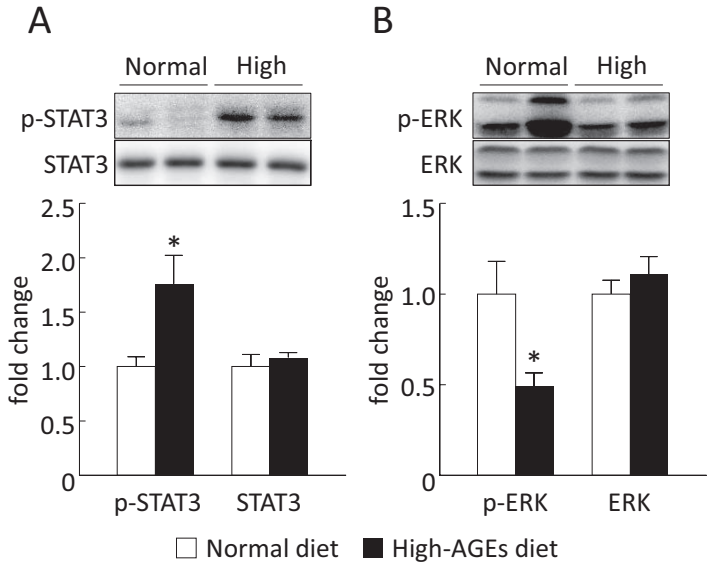

Fig. 5 The effect of AGEs on phosphorylation of STAT3 and ERK in mouse skeletal muscle. Extensor digitorum longus muscles were dissected from mice treated with a normal diet or a diet high in AGEs for 16 weeks, and then analyzed for phosphorylation of signal transducer and activator of transcription 3 (STAT3) Tyr^{705} (p-STAT3) (A) and extracellular signal-regulated kinase (ERK) Thr^{202}/Tyr^{204} (p-ERK) (B). Representative immunoblots are shown. Data are expressed as mean ± SE; n = 9/group. *P < 0.05 vs. normal diet group.

around 75 kDa or over 150 kDa (Fig. 1C). Accordingly, previous studies have shown that incubation with glucose for 7 days generated CML-modified proteins around 65 kDa in macrophages[30], and that osteoblasts treated with glucose-induced AGEs-BSA for 1 day had more CML-modified proteins around 70 kDa or over 200 kDa[31]. Members of the 70 kDa heat shock protein family are suggested to be an AGE-modified protein in hepatic cells[32]. However, further investigation is necessary to clear which proteins are modified by AGEs in skeletal muscle. It is speculated that accumulation of AGE (CML)-modified proteins at high molecular weight (>150 kDa) is due to protein–protein cross-linking and aggregation, which are characteristics of AGEs[33].

STAT3 is a transcription factor that plays a major role in the regulation of the myogenic lineage[34]. STAT3 is phosphorylated at Tyr^{705}, which induces STAT3 dimerization, translocation to the nucleus, and binding of DNA[35]. It has been shown that elevated phosphorylation of STAT3 Tyr^{705} is observed in atrophied skeletal muscle in mice, as occurs in diabetes, chronic kidney disease, or cancer cachexia, and STAT3 ablation partly prevented skeletal muscle loss under these conditions[36,37]. Moreover, it has been reported that STAT3 signaling is elevated in older skeletal muscle satellite cells; and inhibition of STAT3 signaling resulted in increased numbers of satellite cells, facilitated muscle repair, and enhanced functional performance[38]. These studies indicate that STAT3 activation is involved in aging-related skeletal muscle loss. In the present study, we found that AGE treatment in vitro (Fig. 2 and 3A) and in vivo (Fig. 5A) increased STAT3 Tyr^{705} phosphorylation in skeletal muscle, suggesting that AGE-induced inhibition of muscle formation is partly via STAT3 activation. Indeed, it is accepted that STAT3 is a main mediator that interacts with the receptor to induce AGE signaling[39].

ERK, a member of the mitogen-activated protein kinase family, is a critical regulator of myogenesis[40]. The phosphorylation of both ERK Thr^{202} and Tyr^{204} is required for activation of the enzyme[41]. It has been reported that AGE treatment enhanced ERK phosphorylation in rat vascular smooth muscle cells[42], renal fibroblasts[43], and human fetal osteoblastic cells[44]. On the other hand, we found that AGE treatment in skeletal muscle cells decreased ERK Thr^{202}/Tyr^{204} phosphorylation (Fig. 2 and 3B), and that long-term AGE intake also reduced phosphorylation in rat skeletal muscle (Fig 5B). This is the first report to show the AGE-induced ERK modification in skeletal muscle. Previous reports have shown that ERK is involved in the differentiation of skeletal muscle cells[45], and that the ERK signaling diminished with aging in human skeletal muscle[46]. Therefore, downregulation of ERK signaling may be a factor in AGE-mediated phenotype changes in skeletal muscle.

Insulin/IGF-1 signaling is closely linked to maintaining skeletal muscle mass. Impaired insulin/IGF-1 signaling causes abnormal protein metabolism, and thereby results in muscle wasting in several catabolic conditions, including chronic kidney disease[47], diabetes[48], cancer cachexia[49], and aging[50]. IGF-1 levels are also lower in aged men compared to young men[51]. Downregulation of insulin/IGF-1 signaling results in a reduction in the activity of PI3K, Akt, mammalian target of rapamycin (mTOR), p70S6K, and eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1) in aged skeletal muscle[52], which causes muscle protein degradation. A recent report showed that AGE treatment in skeletal muscle cells impaired insulin signaling by decreasing the phosphorylation of Akt Ser^{473} [15,17]. Furthermore, the previous studies by our group and others[16,18] have demonstrated that long-term AGE intake in rodents reduced the phosphorylation of Akt Ser^{473} and p70S6K Thr^{389}. In the present study, it was shown that the phosphorylation level of almost all of the phosphorylation sites in insulin/IGF-1 signaling was

decreased (Fig. 4A). Taken together, it is suggested that insulin/IGF-1 signaling is downregulated by AGEs, and this may contribute to aging-related skeletal muscle loss by inhibiting protein synthesis and accelerating protein degradation.

In conclusion, the present study showed the following novel findings related to the effects of AGEs on molecular responses in skeletal muscle. First, treating C2C12 cells with AGEs promoted AGE accumulation and suppressed myotube formation. Second, AGEs impacted the phosphorylation of multiple signaling molecules involved in regulating muscle cell size. Upregulation of STAT3 phosphorylation and downregulation of ERK phosphorylation were observed in AGE-treated C2C12 cells and mouse skeletal muscle. Insulin/IGF-1 signaling was also downregulated by AGEs. These findings suggest that the cellular signal transduction pathways in skeletal muscle are impaired by AGEs. It is speculated that the progress of sarcopenia is partly attributable to AGE-mediated dysfunctions of cellular signal transduction systems.

Conflict of Interests

The authors have declared that no conflict of interests exists.

Acknowledgments

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