Blunting of AICAR-induced human skeletal muscle glucose uptake in type 2 diabetes is dependent on age rather than diabetic status

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We reported previously that an in vivo infusion with 5-aminoimidazole-4-carboxamide-1β-d-ribofuranoside (AICAR) acutely stimulates skeletal muscle 2-deoxyglucose (2DG) uptake in healthy young men, doubling its rate of uptake after 3 h (6). Despite evidence that, in isolated human muscle strips, AICAR activates AMP kinase (AMPK) activity and increases 2DG uptake (16), the stimulatory effect on 2DG uptake that we observed was associated not with increased AMPKα1 or AMPKα2 activity but with increased ERK1/2 phosphorylation (6). During the AICAR infusion, the increase in extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation was insulin independent, as evidenced by a lack of change in either the circulating insulin concentration or the phosphorylation of Akt on Ser473, a key intermediary of the insulin-signaling pathway (6).

The role of ERK1/2 signaling in regulating skeletal muscle glucose uptake is poorly defined compared with that of insulin or AMPK signaling. In cultured L6 cells, ERK1/2 signaling has been shown, at least partly, to mediate the stimulation of glucose transporter-4 (GLUT4) translocation and glucose uptake by AICAR through sequential activation of its upstream kinase MAPK kinase 1/2 and ERK1/2 and via downstream activation of phospholipase D, phosphatidic acid, and atypical PKCs (2). Furthermore, pharmacological or genetic inhibition of any of the components of the ERK pathway abolished the AICAR-induced stimulation of glucose uptake. These observations support a putative role for ERK1/2 in regulating skeletal muscle glucose transport.

Much evidence supports the proposition that a variety of molecular signaling defects are associated with obesity, aging, and type 2 diabetes mellitus and may underlie insulin resistance. With respect to the AMPK pathway, although it has previously been suggested that the expression and activity of the skeletal muscle AMPK pathway is similar in subjects with type 2 diabetes and in healthy people (14, 18), a recent study by Sriwijitkamol et al. (24) suggests that dysfunction in the AMPK pathway may exist in obesity and type 2 diabetes. Those authors showed a marked blunting of muscle AMPKα2 activation during low- and moderate-intensity cycling and AS160 phosphorylation during moderate-intensity cycling in the obese and obese diabetic subjects compared with healthy controls (24). Furthermore, with respect to ERK signaling, there is blunting of the increase in ERK1/2 phosphorylation after resistance exercise in older compared with young subjects (although a higher postabsorptive phosphorylation) (27).

Therefore, we sought to determine the acute effects of AICAR on muscle 2DG uptake in type 2 diabetes mellitus patients and in healthy and age- and body mass index (BMI)-matched old men compared with those previously reported in...
METHODS

Subject Characteristics

Six healthy young men (age 23 ± 3 yr, weight 77 ± 5 kg, BMI 25 ± 2 kg/m²), eight older men (age 59 ± 4 yr, weight 84 ± 4 kg, BMI 28 ± 2 kg/m²), and eight men with type 2 diabetes (age 62 ± 4 yr, weight 80 ± 5 kg, BMI 27 ± 2 kg/m²) participated in the study. Six of the patients were diet controlled, and two received oral hypoglycemic agents only; these drugs were withheld from them the evening before the study. The subjects were habitually active at a recreational level but were instructed to refrain from strenuous physical activity for 2 days prior to the study and to adhere to their usual diet. The subjects were informed of the experimental protocol both verbally and in writing before they gave informed consent. The study protocol was approved by the Tayside Ethics Committee and conducted in accordance with the Helsinki Declaration.

Experimental Protocol

The experimental protocol and the results from the young subjects have already been described (6), and new subjects were not recruited specifically for this component of the study. The protocol for the older and type 2 diabetic subjects was carried within 12 mo of the earlier study specifically to determine the effects of aging and type 2 diabetes. The subjects attended the laboratory having fasted overnight specifically for this component of the study. The protocol for the older individuals was conducted in accordance with the Helsinki Declaration.

Analytical Methods

Materials. Except where otherwise stated, all chemicals were of the highest quality available from Sigma-Aldrich (Poole, UK) and AICAR (Toronto Research Chemicals, Toronto, ON, Canada) and the other for blood sampling. 2DG was administered as a primed, constant infusion (priming dose 10 mg/kg, infusion rate of 6 mg·kg⁻¹·h⁻¹) throughout the study, with AICAR given from 3 to 6 h. All biopsies were taken through separate incisions made from distal to proximal areas of the quadriceps. During the infusions, subjects were given free access to water but remained recumbent.

Muscle 2DG and 2DG-6-phosphate. Frozen muscle biopsy samples (30–40 mg) were ground in liquid nitrogen, and the frozen powder was transferred to 70% ethanol. The sample was vortex mixed and then centrifuged at 5,000 g for 10 min. The supernatant was used for 2DG/2DG-6-phosphate analysis, as described previously (6). Calculation of glucose uptake was performed as described previously (6). Total 2DG uptake is given as mean absolute values and the mean fold change for AICAR stimulation from individual basal 2DG uptake rates.

Muscle 5-aminoimidazole-4-carboxamide-1-β-D-ribosyl monophosphate and adenosine nucleotides. Muscle 5-aminoimidazole-4-carboxamide-1-β-D-ribosyl monophosphate (ZMP) and adenosine nucleotide concentrations were determined from perchloric acid extracts using capillary electrophoresis, as described previously (6).

Total AMPKα isofrom-specific activity. Muscle lysates were prepared by homogenization of muscle tissue (1:20, wt/vol) in a buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 20 mM Na-pyrophosphate, 20 mM β-glycerophosphate, 10 mM NaF, 2 mM Na-orthovanadate, 2 mM EDTA, 1% Nonidet P-40, 10% glycerol, 2 mM PMSF, 1 mM MgCl₂, 1 mM CaCl₂, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 3 mM benzamidine. Lysates were rotated end over end for 1 h at 4°C and then cleared by centrifugation at 17,500 g at 4°C for 1 h. Protein in the supernatants was measured by the bicinchoninic acid (BCA) method (Pierce, Rockford, IL). AMPKα isofrom-specific activity was measured in immunoprecipitate from 100 μg of muscle lysate protein by use of antibodies raised against the α1 and α2 subunits bound to protein G-Sepharose beads. A PS1 filter paper assay, using AMARA peptide (200 μM) as substrate and [32P]ATP, was used to measure AMPK activity in the presence of saturating AMP concentration (0.2 mM) (14).

Western Blotting

Need adding for new blots. The level and phosphorylation of ERK1/2 was determined by Western blot using phospespecific antibodies. Samples were homogenized in 9 vol of buffer containing 20 mM Tris, 100 mM KCl, 10 mM NaF, 1 mM EDTA, 20 mM β-glycerophosphate, 25 mM Na₃VO₄, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml phenylmethylsulfonyl fluoride. Protein concentration was quantified using the BCA assay system (Pierce). Equal aliquots of samples were resolved by SDS-PAGE, transferred to nitrocellulose membranes, blocked with 5% milk in Tris-buffered saline with 1.0% Tween-20, and then probed with the appropriate antibodies. Phosphospecific antibody to ERK1/2 (P-Thr202/Tyr204, 1:1,000), phoshospecific AMPK (P-T172, 1:1,000), phoshospecific acetyl-CoA carboxylase (ACC; P-S59, 1:1,000), total AMPK (1:1,000), and total ACC (1:1,000) were from Cell Signaling Technology (Beverly, MA), and β-actin (1:5,000) was purchased from Sigma. After phosphospecific analysis, membranes were stripped with 1× Western Re-Prosbe (Oncogene Research Products, San Diego, CA) and reprobed. Protein bands were scanned and quantified by densitometry using AIDA Image Analyzer software.

Analysis of AS160 phosphorylation was done by immunoprecipitation of AS160 from the cell lysate prior to Western blot analysis. Briefly, 4 μg of antibody/mg of lysate was mixed at 4°C for 1 h, and then protein G-Sepharose (30 μl of a 50% suspension in lysis buffer) was added and mixed for an additional 1 h. Phosphospecific AS160 (P-S588, 1:1,000) was raised against CMRGLGpSVDSFER (cysteine + residues 582–594, phospho-Ser) (38), and total AS160 (1:1,000) was raised against KAKIGNKP (near the COOH terminus of human AS160). Data and statistical analysis. Data are expressed as means ± SE. Results were compared using a paired t-test or a repeated-measures ANOVA with Bonferroni posttest where there were three or more data sets. The null hypothesis was rejected at the 5% level (P < 0.05).
Table 1. Plasma concentrations of glucose, 2DG, and insulin at baseline, after 3 h of 2DG and at 6 h, after a 3-h AICAR infusion (10 or 20 mg·kg\(^{-1}·h^{-1}\)) in type 2 diabetes (n = 8), in age-matched healthy (nondiabetic) controls (older; n = 8), and in young (nondiabetic) controls (young; n = 6).

<table>
<thead>
<tr>
<th>AICAR dose, mg·kg(^{-1}·h^{-1})</th>
<th>Study Time, h</th>
<th>Glucose, mmol/l</th>
<th>2DG, µmol/l</th>
<th>Insulin, mIU/l</th>
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</thead>
<tbody>
<tr>
<td>10</td>
<td>0</td>
<td>5.02±0.13</td>
<td>349±49</td>
<td>7.6±0.7</td>
</tr>
<tr>
<td>Young</td>
<td>3</td>
<td>6.73±0.23</td>
<td>248±10</td>
<td>11.1±0.9</td>
</tr>
<tr>
<td>Older</td>
<td>6</td>
<td>6.75±0.51</td>
<td>254±48</td>
<td>12.7±1.5</td>
</tr>
<tr>
<td>Type 2 diabetes</td>
<td></td>
<td>7.23±0.80*</td>
<td>282±12</td>
<td>13.0±2.7</td>
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<tr>
<td>20</td>
<td>0</td>
<td>5.32±0.15</td>
<td>268±36</td>
<td>11.5±1.8</td>
</tr>
<tr>
<td>Young</td>
<td>3</td>
<td>6.58±0.60</td>
<td>254±48</td>
<td>12.2±1.2</td>
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<tr>
<td>Older</td>
<td>6</td>
<td>6.58±0.51</td>
<td>254±48</td>
<td>11.8±2.0</td>
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<tr>
<td>Type 2 diabetes</td>
<td></td>
<td>7.32±0.80*</td>
<td>12.2±1.2</td>
<td>13.2±2.9</td>
</tr>
</tbody>
</table>

Values are means ± SE; 2DG, 2-deoxyglucose; AICAR, 5-aminimidazole-4-carboxamide-1-β-D-ribofuranoside. *P < 0.05 vs. young and older.

RESULTS

Plasma: Glucose, 2DG, and Insulin Concentrations

Plasma glucose, 2DG and insulin remained constant throughout all groups (Table 1). Glucose was significantly higher in the subjects with type 2 than in the nondiabetic groups (P < 0.05). There was no significant difference in plasma 2DG or insulin concentration between groups.

Muscle: Metabolite and ZMP concentration

The intramuscular content of ZMP, the intracellular metabolite of AICAR, was measured in muscle samples from healthy older people and patients with type 2 diabetes (Table 2). As expected, ZMP content increased more after the higher vs. lower dose of AICAR (P < 0.05 between doses for the older people) but did not differ between the older people and type 2 diabetes patients at either the lower dose (20 ± 3 vs. 23 ± 3 µmol/kg muscle wet wt) or the higher dose (57 ± 11 vs. 47 ± 11 µmol/kg muscle wet wt), respectively. In the young, we previously calculated the ZMP concentration on the basis of dry weight (6); however, assuming 77% of muscle weight is water (26), then ZMP concentration in the young was 16 ± 3 µmol/kg muscle wet wt. Making the same assumptions for ATP, ADP, and AMP/inosine monophosphate (IMP) in the young, then the respective concentrations equate to 5.014 ± 1.035, 759 ± 115, and 23 ± 10 µmol/kg muscle wet wt. The intramuscular content of ATP, ADP, and AMP/IMP remained constant throughout the studies, with no effect of AICAR on ATP, ADP, and AMP/IMP concentration within muscle in any group. There was also no significant difference in ATP, ADP, and AMP/IMP concentration between groups.

2DG Uptake

Absolute rates of 2DG uptake. Basal 2DG uptake was less in older men than young men and less again in men with type 2 diabetes, although the differences were not statistically significant (Fig. 1). In response to stimulation with a low dose of AICAR, the absolute rate of 2DG uptake increased significantly only in the young (P < 0.05), with no significant response in older people or in type 2 diabetes. The high dose of AICAR did not significantly stimulate further increases in 2DG uptake in older people or in those with type 2 diabetes.

Table 2. Skeletal muscle (intracellular) concentrations of ATP, ADP, AMP/IMP, and ZMP in response to AICAR (10 or 20 mg·kg\(^{-1}·h^{-1}\)) in patients with type 2 diabetes (n = 8), in age-matched healthy (nondiabetic) controls (older; n = 8), and in young (nondiabetic) controls (young; n = 4).

<table>
<thead>
<tr>
<th>AICAR dose, mg·kg(^{-1}·h^{-1})</th>
<th>Study time, h</th>
<th>ATP, mmol/kg wet wt</th>
<th>ADP, µmol/kg wet wt</th>
<th>AMP/IMP, µmol/kg wet wt</th>
<th>ZMP, µmol/kg wet wt</th>
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<tr>
<td>10</td>
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<td>5.01±0.03</td>
<td>759±115</td>
<td>5.56±0.85</td>
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<td>695±54</td>
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<tr>
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<td>3.72±0.34</td>
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</tr>
<tr>
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<td>3.45±0.21</td>
<td>540±1.8</td>
<td>4.29±0.32</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>0</td>
<td>3.58±0.36</td>
<td>557±71</td>
<td>4.38±0.68</td>
<td></td>
</tr>
<tr>
<td>Young</td>
<td>3</td>
<td>3.45±0.21</td>
<td>540±1.8</td>
<td>4.29±0.32</td>
<td></td>
</tr>
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<td>4.29±0.32</td>
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</tbody>
</table>

Values are means ± SE; there were no significant differences between groups. IMP, inosine monophosphate; ZMP, 5-aminimidazole-4-carboxamide-1-β-D-ribofuranoside. *Calculated from published dry weights, assuming 77% water content in skeletal muscle.
There was no difference in basal uptake, so groups are shown as grand means.

**DISCUSSION**

We have demonstrated that the stimulatory effect of an acute 3-h AICAR infusion on the rate of skeletal muscle 2DG uptake is attenuated in older men and in male patients with type 2 diabetes compared with that seen previously in young men (6). In an attempt to overcome this attenuation, we doubled the rate of AICAR infusion from 10 to 20 mg·kg⁻¹·h⁻¹, but only a small increase in additional muscle 2DG uptake resulted. The rise in muscle 2DG uptake seen at 3 h at either rate of AICAR infusion was associated with increased ERK1/2 phosphorylation but not with any change in total AMPKα isoform activity.

We have previously used a primed, constant infusion of 2DG to determine the rate of muscle glucose uptake in healthy young men. This method was validated by taking sequential muscle biopsies and demonstrating that, with steady-state plasma 2DG concentrations, there is a linear incorporation of 2DG into muscle over 9 h (6). In the current study, we applied this method to measure muscle 2DG uptake in older people and in patients with type 2 diabetes. As in the healthy young, we found considerable variability in the rates of basal and AICAR-stimulated 2DG uptake in older people and those with type 2 diabetes. We found that the basal rate of muscle 2DG uptake was reduced in older men with and without type 2 diabetes compared with the young. In response to an acute 3-h AICAR infusion, the rise in 2DG uptake was significantly blunted in older people and patients with type 2 diabetes compared with the healthy young, with the doubling of the amount of AICAR given unable to overcome this blunted stimulation. Thus, the reduction in the basal and the AICAR-stimulated rate of muscle 2DG uptake would seem to be dependent on age rather than diabetic status. An age-dependent decrease in resting skeletal muscle glucose uptake has been demonstrated in rats, with a 37% decrease in basal glucose uptake (25). In humans, insulin sensitivity (as assessed during an intravenous glucose tolerance test) has been shown to decrease by 8% per decade (23).

The differential rates of muscle 2DG uptake between the young, old, or type 2 diabetic men could not be explained by differences in rates of AICAR delivery, uptake of AICAR into muscle, or its intracellular conversion to ZMP between the groups, because similar intramuscular concentrations of ZMP were present in the muscle of all patient groups. Furthermore, the doubling of the AICAR dose was associated with a doubling of the concentration of intramuscular ZMP in the older men and men with type 2 diabetes mellitus.

The finding of reduced skeletal muscle glucose transport in type 2 diabetes is well documented (20), although Koistinen et al. (16) reported that AICAR could significantly increase glucose transport and cell-surface GLUT4 content using ex vivo skeletal muscle strips (albeit slightly less than in healthy controls). We report relative changes in 2DG uptake with AICAR in type 2 diabetes patients very similar to those observed by Koistinen et al. (16); in patients of a similar age to those in the current study, the 2DG uptake increased 2.9-fold in patients with type 2 diabetes compared with that seen previously in young men (6).
and 1.8-fold in control subjects and in subjects with type 2 diabetes, respectively. Also, in aged rats, AICAR administration for 1 wk is only able to increase 2DG uptake 1.2-fold above nontreated levels (25).

With regard to the effects of aging, there is controversy as to whether muscle from elderly subjects has an intrinsic defect in its capacity to transport glucose or whether muscle insulin resistance is a secondary phenomenon explained by altered body composition (10). Skeletal muscle accounts for ∼80% of whole body glucose uptake after exercise or insulin stimulation, and yet, although muscle mass falls with aging, differences in insulin sensitivity between young and old people persist when adjustments are made for differences in lean body mass, suggesting that muscle mass is not the primary determinant of insulin sensitivity (11). Despite the clear association between insulin resistance and visceral fat mass in older people (1) and the higher accumulation of visceral fat in older people than young people (5), intra-abdominal fat mass can account for only about one-half of the observed variation in insulin sensitivity in older people (3). These findings suggest that there are contributory factors to age-related insulin resistance other than simply body composition. Although we did not determine body composition or total muscle mass in the current study, our measurements of 2DG uptake are expressed per kilogram of wet weight of muscle. Considering there is a progressive reduction in muscle mass with aging (7, 22), our finding of reduced muscle glucose uptake per unit of muscle mass in older people suggests that the total deficit in whole body glucose uptake in older people may be even greater than these results suggest.

What is the mechanism by which AICAR stimulates glucose uptake into skeletal muscle? AICAR is a known pharmacological activator of AMPK through which muscle glucose uptake increases. The surprising lack of stimulation of skeletal muscle AMPK isoform activity (of either total AMPK isoform or the individual α-isoforms 1 and 2) in response to an acute AICAR infusion, after either 20 or 180 min, has been addressed in discussing our previous studies in healthy young men (6). We demonstrated that total activity of AMPK 1 and -2 isoforms was unchanged in skeletal muscle after 20 min and 3 h of infusion, although we did detect activation of AMPK immediately after exercise. Therefore, in this study, as in the young men, where we could not detect any change in total AMPK activity, after 3 h of AICAR infusion, we believed it would be unlikely to be different from what we had seen previously (6). In the current study, in older people and in type 2 diabetes, a doubling of the dose of AICAR infusion from 10 to 20 mg·kg⁻¹·h⁻¹ was not able to elicit any increase in total AMPK activity above basal at 3 h. Considering the overwhelming evidence that AMPK
mediates glucose uptake, it may be likely that activation of AMPK is only transient or that the magnitude of the rise was beyond the sensitivity of our current techniques rather than the effect being AMPK independent.

In healthy young men, we have shown that AICAR rapidly induces ERK1/2 phosphorylation (6). This was independent of any change in the insulin-signaling pathway, with no change in Akt phosphorylation during the AICAR infusion (6). Others have shown that AICAR can activate ERK1/2. In rodent muscle and in L6 myoblasts, AICAR-mediated stimulation of glucose uptake was dependent on activation of the ERK1/2 at Thr202/Tyr204, resulting in increased GLUT4 translocation (4). When ERK1/2 is inhibited, using PD-98059, there is a reduction in glucose uptake by 25% compared with that in control animals during moderate electrical stimulation of hindlimb muscle in young rats (21). Supporting our findings of a lower responsiveness of ERK1/2 in older muscle, ERK1/2 phosphorylation in response to high-frequency electrical stimulation was markedly blunted in aged rats compared with young rats immediately postexercise (19).

AMPK is likely to be one of a number of regulators of insulin-independent glucose uptake in muscle (15, 28), with emerging evidence for additional insulin- and AMPK-dependent pathways mediating muscle glucose uptake. However, in the absence of parallel in vitro experiments using specific kinase inhibitors, the results of the current and previous studies are not able to shed any further light on the relative AMPK-dependent or -independent contributions to muscle glucose transport or determine whether the phosphorylation of ERK1/2 induced by AICAR was downstream of AMPK activation (which could not be detected) or whether ERK1/2 signaling was activated in an AMPK-independent manner.

A second study also examining the acute in vivo effects of AICAR in older type 2 diabetes patients (age 64 ± 2 yr) was very recently published by Boon et al. (2). This group infused AICAR for 120 min at a dose 4.5-fold higher than that used in the current and previous study and plasma glucose kinetics determined. Although the rate of glucose appearance decreased (through inhibition of glucose output from the liver), the rate of glucose disappearance (due to peripheral glucose uptake) was unchanged between the AICAR and the control infusion, although the insulin concentration significantly increased. The effects of AICAR on the skeletal muscle were not accompanied by any significant changes in AMPK phosphorylation or α-isofrom activity, although ACC phosphorylation was significantly increased. It is noteworthy that our original study examining the effects of AICAR was carried out in young men where the stimulatory effect of AICAR was preserved. In the current study in men with and without type 2 diabetes (of a similar age to the men studied by Boon et al. (2)), the stimulatory effect of AICAR on muscle glucose uptake was significantly blunted. Furthermore, our measurements of glucose uptake were of skeletal muscle specifically rather than the whole body measurements made by Boon et al. (2) with stable isotopes. Although skeletal muscle represents the majority of whole body glucose uptake, uptake in other tissues, such as adipose tissue, is also accounted for with measurement of glucose kinetics.

It is possible that AICAR is exerting its effects on glucose uptake through other mechanisms besides AMPK activation, although we have no evidence to support such a conclusion. AICAR has been shown to be a vasodilator in animals (12, 13) and may work by increasing blood flow with a blunted effect in the elderly, although this was not measured in the current study.

Finally, the AICAR infusion was well tolerated and associated, with no reported side effects in ours or other studies (9, 17) despite the high dose of AICAR given in the present study being greater than has been given previously in human metabolic studies.

The results of our work suggest that aging skeletal muscle, under postabsorptive conditions, has a reduced capacity for glucose uptake similar to that seen with muscle from patients with type 2 diabetes and that the stimulatory effects of AICAR on muscle glucose uptake are blunted in older men, irrespective of type 2 diabetes, compared with younger men. These findings are physiologically relevant to understanding why aging is associated with an increased prevalence of type 2 diabetes but also suggest that AICAR, at least acutely and at
this dose, has only a limited therapeutic effect on older type 2 diabetic skeletal muscle.

GRANTS

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