Original paper

Rosmarinic Acid Regulates Fatty Acid and Glucose Utilization by Activating the CaMKK/AMPK Pathway in C2C12 Myotubes

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Rosmarinic acid (RA), a polyphenolic compound abundantly found in the *Lamiaceae* family, has been shown to possess antioxidant and anti-inflammatory properties. In this study, we investigated whether it can promote energy expenditure in skeletal muscle cells. RA increased fatty acid oxidation and glucose utilization in C2C12 myotubes in a dose-dependent manner and increased the expression of Ca^{2+} /calmodulin-dependent protein kinase kinase (CaMKK) and activated AMP-activated protein kinase (AMPK). CaMKK inhibitor attenuated the RA-induced AMPK activation and energy expenditure. Sirtuin1 (SIRT1) expression was enhanced by RA at both the gene and protein levels. We also observed that RA enhanced deacetylation of peroxisome proliferator-activated receptor γ coactivator-1 α (PGC1 α), accounting for the increase in energy expenditure-related genes. SIRT1 inhibitor attenuated the RA-induced PGC1 α deacetylation and energy expenditure. Our findings suggest that CaMKK and SIRT1 play a role in the beneficial effects of RA on energy expenditure in skeletal muscle cells.

Keywords: rosmarinic acid, C2C12 myotubes, fatty acid oxidation, glucose utilization, Sirtuin1

Introduction

Skeletal muscle is a major organ involved in mitochondrial oxidative metabolism of energy and therefore plays a central role in whole-body energy homeostasis (Blaak, 2005). In the resting state, fatty acid oxidation in skeletal muscle can account for 90% of the total energy demand of this tissue (Zhang *et al.*, 2010). Fatty acid oxidation in mitochondria is the principal pathway for the metabolism of fatty acids. A decrease in respiration rate and mitochondria biogenesis accounts for a defective energy expenditure, which predisposes to metabolic syndrome, atherosclerosis, cardiomyopathy and diabetes mellitus (Garbarino and Sturley, 2009; Murea *et al.*, 2010; Nair, 2005). Although fatty acids and glucose are important sources of energy in skeletal muscle, excessive amounts of fatty acids and glucose in human blood can cause insulin resistance, leading to the development of diabetes mellitus (Koves *et al.*, 2008; Rossetti *et al.*, 1990). Therefore, it is possible that acceleration of energy expenditure in

skeletal muscle contributes to improvement of metabolic disorders.

During the fatty acid oxidation process, AMP-activated protein kinase (AMPK), Sirtuin1 (SIRT1) and peroxisome proliferator-activated receptor γ coactivator-1 α (PGC1 α) play key roles as metabolic regulators that increase energy expenditure. AMPK is activated in response to an increased AMP/ATP ratio. Alternatively, liver kinase B1 (LKB1) and Ca^{2+} /calmodulin-dependent protein kinase kinase (CaMKK) also activate AMPK by phosphorylating Thr-172 of its α subunit (Hawley *et al.*, 1995; Woods *et al.*, 2003). Activated AMPK phosphorylates and inactivates acetyl coenzyme A carboxylase (ACC), which provides the substrate for fatty acid synthesis, and then promotes energy expenditure. Currently, the evidence suggests that AMPK and SIRT1 display reciprocal interactions with each other (Canto *et al.*, 2009; Ivanov *et al.*, 2008; Lan *et al.*, 2008). SIRT1 is a member of the class III NAD^+ -dependent histone deacetylases and is predominantly localized in the nucleus (Gerhart-Hines *et al.*,

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2011). SIRT1 can activate PGC1 α by deacetylation, resulting in increased fatty acid levels and glucose utilization (Blander and Guarente, 2004). Therefore, identification of a compound that activates the AMPK and SIRT1 pathways would significantly contribute to our ability to treat obesity and type 2 diabetes by accelerating energy expenditure.

Recently, a number of compounds from natural sources have been reported to activate AMPK, such as resveratrol (Park *et al.*, 2012; Price *et al.*, 2012), naringenin (Zygmunt *et al.*, 2010) and genistein (Palacios-González *et al.*, 2014). In particular, resveratrol also activates SIRT1 (Price *et al.*, 2012). To identify a novel compound that harbors anti-diabetes and anti-obesity potential, we evaluated the pharmacological effects of a number of natural single compounds and found that rosmarinic acid (RA) accelerated energy expenditure in skeletal muscle cells. RA is a polyphenolic compound and the main constituent of the Lamiaceae family of plants, which includes perilla, basil, rosemary and sage. Several biological activities have been described for RA, such as anti-oxidative, anti-inflammatory, anti-mutagenic and neuroprotective activities (Alkam *et al.*, 2007; Ly *et al.*, 2006). Rosemary extract, containing RA, has been reported to induce glucose consumption via activation of AMPK signaling (Kim *et al.*, 2009; Naimi *et al.*, 2015; Tu *et al.*, 2013). However, little is known about the effect of RA on accelerating energy expenditure in skeletal muscle cells and the mechanisms underlying this process. In this study, we explored whether RA can modulate the metabolism of fatty acids and glucose in C2C12 skeletal muscle cells. We then investigated whether RA affects AMPK phosphorylation, SIRT1 expression and PGC1 α deacetylation.

Materials and Methods

Reagents RA (see chemical structure in Fig. 1A) and gentamicin were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). C2C12 cells were from DS Pharma Biomedical Co., Ltd. (Osaka, Japan). Bovine serum albumin (BSA) was obtained from Medical & Biological Laboratories (Nagoya, Japan). Fetal bovine serum was from Biological Industries (Beth Haemek, Israel). Horse serum was from Life Technologies (Gaithersburg, MA, USA). ^3H -Palmitate was from Perkin Elmer Japan Co., Ltd. (Tokyo, Japan). FastStart Universal SYBR Green Master (ROX) was from Roche Applied Science (Indianapolis, IN, USA). Antibodies for β -actin, AMPK α , phospho-AMPK α (Thr-172), ACC, phospho-ACC (Ser-79), Akt, phospho-Akt (Thr-308) and acetyl-lysine were from Cell Signaling Technology (Beverly, MA, USA). Antibodies for CaMKK, PGC1 α and SIRT1 were from Abcam Inc. (Cambridge, MA, USA).

Cell culture and treatment C2C12 myoblasts were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 50 $\mu\text{g}/\text{mL}$ gentamicin and 10% fetal bovine serum. To differentiate myoblasts into myotubes, cells were fed DMEM containing 2% horse serum for 4 days. Differentiated

Fig. 1. RA treatment promotes fatty acid oxidation and glucose utilization in C2C12 myotubes.

(A) RA structure. (B) Effect of RA on Fatty acid oxidation. (C) Effect of RA on glucose utilization. Cells were treated with various concentrations of RA, 100 μM L-carnitine and ^3H palmitate (200 μM cold palmitate and 185 kBq/mL ^3H palmitate in each well) for 24 h. The culture media were collected for measurement of palmitate oxidation and glucose concentration. Values are mean $\pm$ SD ($n = 4$). Data are representative of multiple experiments. * $P < 0.05$; ** $P < 0.01$ vs. control.

myotubes were serum-starved for 6 h in serum-free DMEM supplemented with 1% BSA. Subsequently, cells were incubated in serum-free DMEM supplemented with 1% BSA, 100 μM L-carnitine, RA and ^3H -palmitate (mixture of 200 μM cold palmitate and 185 kBq/mL ^3H -palmitate in each well) for 24 h. After incubation, the culture media were collected for measurement of palmitate oxidation and glucose concentration.

Measurement of palmitate oxidation and glucose concentration For measurement of palmitate oxidation, proteins bound to palmitate were removed by trichloroacetic acid precipitation, and the supernatant was loaded into a 96-well filter plate (Unifilter GF/B, GE Healthcare Japan, Tokyo, Japan) containing an equilibrated, activated charcoal slurry. The plate was centrifuged for 10 min at $1,000 \times g$. Following centrifugation, the charcoal-containing plate was discarded and the filtrate was counted using a scintillation counter (Plate Chameleon V, Turk, Finland). For measurement of glucose concentration, collected media were assayed for glucose concentration using a Glucose CII-test kit (Wako Pure Chemical Industries, Ltd.).

Quantitative real-time PCR Total RNA from C2C12 myotubes was isolated using Isogen reagent (Wako Pure Chemical Industries, Ltd.). To obtain cDNA, RNA (1 μg) was reverse transcribed using a PrimeScript RT Reagent Kit (Takara Bio Inc., Shiga, Japan). Quantitative real-time PCR was performed using an ABI 7500 Real-Time PCR System (Life

Technologies) according to the manufacturer's protocol. Primer sequences used in PCR were: *36B4*, 5'-ACTGGTCTAGGACCCGAGAAG-3' and 5'-CTCCACCTTGCTCCAGTC-3'; *CD36*, 5'-TTGTACCTATACTG T G G C T A A A T G A G A - 3 ' and 5'-CTTGTGTTTGAACATTTCTGCTT-3'; *long chain acyl-CoA dehydrogenase (LCAD)*, 5'-GCTTATGAATGTGTGCAACTCC-3' and 5'-CCGAGCATCCACGTAAGC-3'; *uncoupling protein 3 (UCP3)*, 5' - T A C C C A A C C T T G G C T A G A C G - 3 ' and 5' - G T C C G A G G A G A G A G C T T G C - 3 ' ; *SIRT1*, 5' - C A G T G A G A A A T G C T G G C C T A - 3 ' and 5'-TTGGTGGTACAAACAGGTATTGA-3'; and *glucose transporter 4 (GLUT4)*, 5' - C A T G G C T G T C G C T G G T T T C - 3 ' and 5'-AAACCCATGCCGACAATGA-3'. Data analyses were performed using the 7500 System SDS software, version 1.3.1 (Life Technologies).

Western blot analysis Cells were lysed with M-PER mammalian protein extraction reagent (Thermo Scientific, Rockford, IL, USA) supplemented with protease inhibitor cocktail and phosphatase inhibitor cocktail solutions (Wako Pure Chemical Industries, Ltd.). The protein concentration was determined using the BCA protein assay (Thermo Scientific). Equal amounts of protein (20 µg/lane) from each treated group mixed with SDS sample buffer (Wako Pure Chemical Industries, Ltd.) were loaded on a 10% or 12.5% SuperSep Ace gel (Wako Pure Chemical Industries, Ltd.). After electrophoresis, proteins were transferred to a PVDF membrane (Bio-Rad Laboratories, Hercules, CA, USA). The membrane was first blocked in Tris-buffered saline with Tween 20 (TBST) with 4% skim milk or BSA for 1 h, followed by incubation with primary antibodies in TBST with 2% skim milk or BSA for 1 h at room temperature. Blots were washed three times with TBST and then incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. The immunoreactive proteins were visualized using the ECL Plus Western Blotting Detection System (GE Healthcare Japan), and band intensities were quantified with GeneSys/GeneTools software using the GeneGnome-5 Chemiluminescent Imaging System (Syngene, Cambridge, UK).

Immunoprecipitation Immunoprecipitation of PGC1α was carried out using a Dynabeads Protein G Immunoprecipitation Kit (Veritas, Tokyo, Japan) according to the manufacturer's instructions. Briefly, anti-PGC1α antibody was immobilized on Dynabeads Protein G. Then, 500 µg of protein extract were incubated with the Dynabeads-antibody complex for 10 min at room temperature. The Dynabeads-antibody-antigen complex was magnetically isolated. After washing, the immunoprecipitate was eluted in elution buffer. Proteins were separated by SDS-PAGE, and Western blot analysis was performed using anti-PGC1α and anti-acetyl-lysine antibodies.

Statistical analysis Results are expressed as mean ± SD. Statistical analysis was performed by one-way ANOVA followed by Dunnett's post-hoc test using Ekuseru-Toukei 2012 (Social Survey Research Information Co. Ltd., Tokyo, Japan). Values of P

Fig. 2. RA treatment enhances the expression of genes and proteins related to energy expenditure

(A) Effects of RA on the gene expression of *LCAD*, *UCP3* and *GLUT4* in cells treated with RA for 24 h. Data are expressed relative to the control cells after normalization to *36B4*. Values are mean ± SD ($n = 3$). Data are representative of multiple experiments. * $P < 0.05$; ** $P < 0.01$ vs. control. (B) RA effects on the expression of proteins associated with fatty acid oxidation and glucose utilization. Cells were treated with RA for 24 h. The band intensities were quantitated by densitometry and shown as a fold of increase compared to control (right graph). Values are mean ± SD. Analytical details are described in the Materials and Methods.

$P < 0.05$ were considered statistically significant.

Results

RA promotes palmitate oxidation and glucose utilization C2C12 myotubes were treated with RA and palmitate. As shown in Fig. 1B and C, RA promoted fatty acid oxidation and glucose utilization in a dose-dependent manner, which was statistically significant from 25 µM RA. To further characterize the effects of RA on fatty acid oxidation, we examined the mRNA expression of the energy expenditure-related genes, *LCAD*, *UCP3* and *GLUT4*. The mRNA expression of *LCAD*, *UCP3* and *GLUT4* significantly increased in cells treated with 50 µM, 25 – 50 µM and 25 – 50 µM RA, respectively (Fig. 2A). Next, the levels of these proteins were investigated using Western blot analysis. Similar trends were observed with the protein expression of *LCAD*, *UCP3* and *GLUT4* after RA treatment (Fig. 2B). It is known that the expression of *LCAD* and *UCP3* is regulated by *PPARα* and *PPARδ* (Narkar *et al.*, 2008; Rakhshandehroo *et al.*, 2010); however, RA did not exert *PPARα* and *PPARδ* agonist activity (data not shown). Moreover, although glucose uptake into skeletal muscle cells is promoted by insulin-dependent translocation of *GLUT4* through

phosphorylation of Akt, RA did not modulate the phosphorylation of Akt at Thr308 in cells treated for 6 h (Fig. 3A). Therefore, other factors may be involved in fatty acid oxidation and glucose utilization upon RA treatment.

Effects of RA on AMPK activation As AMPK activation can increase energy expenditure in skeletal muscle by phosphorylation and inhibition of ACC, leading to decreased levels of malonyl-CoA (Merrill *et al.*, 1997), we examined whether RA treatment could activate AMPK in C2C12 myotubes. Treatment with RA for 6 h tended to increase the phosphorylation of Thr-172 on the catalytic α subunit of AMPK (Fig. 3A). ACC, an immediate downstream target protein, showed a similar trend of activation (Fig. 3A). Because CaMKK up-regulation activates AMPK, we tested the effects of RA on CaMKK and observed an increasing trend of CaMKK protein (Fig. 3A). To examine whether CaMKK is

(A) mRNA levels of SIRT1 in C2C12 myotubes treated with RA for 24 h. Data are expressed relative to the control cells after normalization to 36B4. Values are mean $\pm$ SD ($n = 3$). * $P < 0.05$; ** $P < 0.01$ vs. control. (B) Protein levels of SIRT1 in C2C12 myotubes treated with RA for 24 h. The band intensities were quantitated by densitometry and shown as a fold of increase compared to control (lower graph). Values are mean $\pm$ SD. Analytical details are described in the Materials and Methods.

(A) Western blot analysis of PGC1 α and acetyl-lysine proteins in C2C12 myotubes treated with RA and/or Ex-527 for 24 h. C2C12 myotubes were pre-treated with Ex-527, a SIRT1 inhibitor, for 30 min, and then treated with RA for 24 h. PGC1 α immunoprecipitates were analyzed by Western blotting using antibodies against PGC1 α and acetyl-lysine antibodies. Data shown are representative of multiple experiments. The band intensities were quantitated by densitometry and shown as a fold of increase compared to control (lower graph). (B) Effect of SIRT1 inhibitor on RA-induced fatty acid oxidation and glucose utilization. C2C12 myotubes were pre-treated with Ex-527, a SIRT1 inhibitor, for 30 min, and then treated with RA for 24 h. Analytical details are described in the Materials and Methods. Values are mean $\pm$ SD ($n = 4$). Data are representative of multiple experiments. * $P < 0.05$; ** $P < 0.01$ vs. control. ## $P < 0.01$ vs. RA 50 μ M.

involved in AMPK activation, fatty acid oxidation and glucose utilization upon RA treatment, we used a selective CaMKK inhibitor (STO-609). STO-609 has an *in vitro* IC₅₀ of 0.05–0.14 µg/mL for CaMKK (Tokumitsu *et al.*, 2002) and has been used at about 2 µg/mL for muscle cells by various studies (Iwanaka *et al.*, 2006; Kim *et al.*, 2016). We also used STO-609 at 2 µg/mL. STO-609 showed an attenuating trend with respect to the phosphorylation levels of AMPK induced by RA (Fig. 3B). Moreover, STO-609 significantly blocked the RA-induced increase in fatty acid oxidation and glucose utilization (Fig. 3C).

Effects of RA on SIRT1 and PGC1 α As AMPK and SIRT1 display reciprocal interactions with each other (Canto *et al.*, 2009; Ivanov *et al.*, 2008; Lan *et al.*, 2008) and activation of AMPK has been reported to increase SIRT1 expression in skeletal muscle (Suwa *et al.*, 2011), we examined whether RA can increase SIRT1 expression in C2C12 myotubes. Treatment of C2C12 myotubes with RA for 24 h resulted in up-regulated gene expression of SIRT1 (Fig. 4A). Similarly, the protein level of SIRT1 showed a trend of increase by RA treatment (Fig. 4B). It is known that SIRT1 deacetylates the lysine-site of PGC1 α and activates downstream energy expenditure (Finck and Kelly, 2006; Lagouge *et al.*, 2006). To determine the effects of RA on deacetylation of PGC1 α in C2C12 myotubes, we carried out an immunoprecipitation assay with anti-PGC1 α antibody, followed by Western blot analysis with anti-acetylated lysine antibody. As expected, acetylation of PGC1 α showed a trend of decrease with RA treatment (Fig. 5A). Next, we used a selective SIRT1 inhibitor (Ex-527) to determine the role of SIRT1 in the RA-induced effects. As Ex-527 has been used at 5–20 µM for muscle cells by various studies (Price *et al.*, 2006; Zygumt *et al.*, 2010), we used Ex-527 at 10 µM. Ex-527 attenuated the deacetylation of PGC1 α and the promotion of fatty acid oxidation and glucose utilization (Figs. 5A and B). These findings indicate that elevation of fatty acid oxidation and glucose utilization after RA treatment may be attributed to the activation of AMPK and SIRT1.

Discussion

Our results demonstrated that RA increased fatty acid oxidation and glucose utilization associated with up-regulating genes and proteins related to energy expenditure in skeletal muscle myotubes. Pharmacological inhibition study indicated that this modulation works by activating the CaMKK/AMPK pathway. Finally, we found that RA increased SIRT1 expression and PGC1 α deacetylation. This study is the first to report the effects and mechanisms of action of RA in energy expenditure, which are mediated through activation of CaMKK/AMPK, SIRT1 and PGC1 α .

RA has been reported to exert anti-oxidative, anti-inflammatory, anti-mutagenic and neuroprotective activities (Ly *et al.*, 2006; Alkam *et al.*, 2007). In particular, RA has been shown to attenuate diabetes-induced inflammation and oxidative stress.

However, little is known about the effects of RA on energy expenditure in skeletal muscle cells. In this study, exposure to RA increased fatty acid oxidation and glucose utilization in C2C12 myotubes. However, RA did not promote Akt phosphorylation or PPAR α and PPAR δ agonist activity, suggesting that the RA-induced enhancement of fatty acid oxidation and glucose utilization is regulated, at least in part, by another signaling pathway.

AMPK is a potent metabolic regulator and plays an important role in various metabolic pathways, such as lipid and glucose metabolism (Merrill *et al.*, 1997). Recently, a number of compounds from natural sources, such as resveratrol (Price *et al.*, 2012; Park *et al.*, 2012), naringenin (Zygumt *et al.*, 2010) and genistein (Palacios-González *et al.*, 2014), have been reported to activate AMPK in skeletal muscle cells. Resveratrol and genistein, in particular, have been reported to increase fatty acid oxidation in skeletal muscle cells via phosphorylation and activation of AMPK; resveratrol has been reported to activate AMPK through increasing CaMKK expression (Park *et al.*, 2012). In this study, there was a trend toward an increase in the phosphorylation levels of AMPK and ACC and the protein levels of CaMKK by RA treatment. More importantly, the CaMKK inhibitor, STO-609, attenuated the RA-stimulated energy expenditure. Collectively, these data suggest that RA promotes fatty acid oxidation and glucose utilization through activation of the CaMKK/AMPK signaling pathway.

SIRT1 plays a key role in regulating lipid metabolism in mammals (Feige *et al.*, 2008). The activation of SIRT1 has been considered as a candidate for ameliorating lipid disorders (Ruderman and Prentki, 2004). Currently, the evidence suggests that AMPK and SIRT1 display reciprocal interactions with each other; AMPK activates SIRT1 by an indirect increase in cellular NAD⁺ levels (Canto *et al.*, 2009); however, SIRT1 deacetylates the AMPK kinase, LKB1, leading to increased phosphorylation and activation of AMPK (Ivanov *et al.*, 2008; Lan *et al.*, 2008). Moreover, it is known that SIRT1 deacetylates the lysine site of PGC1 α , a transcriptional co-regulator, and activates downstream energy expenditure via induction of its related genes (Finck and Kelly, 2006; Lagouge *et al.*, 2006). In this study, RA increased SIRT1 expression at the gene and protein levels and deacetylated PGC1 α . These changes resulted in up-regulation of LCAD, UCP3 and GLUT4. As LCAD is a key enzyme in mitochondrial β -oxidation (Chegary *et al.*, 2009) and UCP3 is the rate-limiting enzyme in mitochondrial uncoupling (Bezaire *et al.*, 2005), their increase may play a role in promotion of fatty acid oxidation induced by RA. Moreover, as GLUT4 plays a role in glucose homeostasis by allowing glucose to enter muscle cells (Huang and Czech, 2007), up-regulation of GLUT4 may contribute to the promotion of glucose utilization. To establish whether SIRT1 is required for RA-induced energy expenditure, Ex-527, a selective SIRT1 inhibitor, was used to treat the cells in combination with RA. Ex-527 attenuated the ability of RA to induce PGC1 α deacetylation, fatty acid oxidation and glucose utilization in C2C12

myotubes. These observations are consistent with a previous report, in which SIRT1-knockout blocked resveratrol-induced fatty acid oxidation in skeletal muscle (Price *et al.*, 2012).

Perilla leaf extract has been shown to ameliorate obesity and dyslipidemia induced by a high-fat diet (Kim and Kim, 2009). In perilla leaf, RA, luteolin, and apigenin are representative bioactive components, and RA is the predominant compound. Luteolin has been reported to enhance glucose consumption with activation of AMPK in adipocytes (Xiao *et al.*, 2014). Rosemary extract, containing RA and carnolic acid, has been reported to increase glucose consumption via activation of AMPK signaling in HepG2 hepatic cells and in skeletal muscle cells (Tu *et al.*, 2013, Naimi *et al.*, 2015). Carnolic acid has been reported to activate AMPK in skeletal muscle (Lipina and Hundal, 2014). However, little is known about the effect of RA on glucose consumption via activation of AMPK signaling. The consistency between previous reports of extracts and our results indicates that RA may, at least in part, be involved in the beneficial effects of the extract of Lamiaceae family plants.

Finally, this study suggests that RA can enhance fatty acid oxidation and glucose utilization in C2C12 myotubes by activating key regulators of the energy-sensing network, such as AMPK, SIRT1 and PGC1 α . With regard to AMPK activation, we think that CaMKK expression is induced by RA treatment, followed by AMPK signal activation. With regard to SIRT1 and PGC1 α , we propose that RA treatment increases both gene expression and protein levels of SIRT1, followed by PGC1 α deacetylation and induction of its downstream target genes. Both CaMKK inhibitor and SIRT1 inhibitor attenuated the RA-induced energy expenditure, indicating that CaMKK and SIRT1 play a role in the beneficial effects of RA on energy expenditure in skeletal muscle cells. These RA effects on C2C12 skeletal muscle cells may provide the basis for improving metabolic disorders.

Conclusions

In conclusion, our findings suggest that RA promotes energy expenditure associated with up-regulating genes and proteins related to energy expenditure in skeletal muscle cells. In particular, we observed that RA activated the CaMKK/AMPK pathway. Moreover, RA increased SIRT1 expression and deacetylation of PGC1 α . These effects of RA on skeletal muscle cells may provide the basis for improving metabolic disorders.

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