Beraprost sodium, a stable prostacyclin analogue, improves insulin resistance in high-fat diet-induced obese mice

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Abstract

Obesity induces hypertrophy of adipocyte resulting in production of pro-inflammatory cytokines such as tumor necrosis factor- α (TNF- α) and monocyte chemoattractant protein 1 (MCP1 (CCL2)). These cytokines play an important role in the development of insulin resistance. Beraprost sodium (BPS), a prostaglandin I₂ analogue, is reported to attenuate inflammation. In this study, we examined the effect of BPS on glucose metabolism in mice fed a high-fat diet (HFD). Four-week-old C57/B6 male mice were fed a HFD for 12 weeks (HFD group) and the treatment group received oral BPS (300 μ g/kg per day) for the same period. Then, glucose metabolism, histological changes, and gene expression of white adipose tissue (WAT) were examined. Body weight was increased, and glucose

intolerance and insulin resistance were developed in the HFD group. Treatment with BPS improved glucose tolerance and insulin action without body weight change. Histological analysis of WAT showed an increase in the size of adipocyte and macrophage infiltration in the HFD group, which was attenuated by BPS treatment. BPS reduced HFD-induced expression of MCP1 and TNF- α in WAT. BPS also attenuated hepatic steatosis induced by the HFD. These results suggest that BPS improved glucose intolerance possibly through suppression of inflammatory cytokines in WAT. BPS may be beneficial for the treatment of obesity-associated glucose intolerance.

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Introduction

Obesity plays a central role in the development of metabolic syndrome (Wajchenberg 2000), a constellation of risk factors such as insulin resistance, dyslipidemia, and high blood pressure. Accumulation of visceral fat rather than subcutaneous fat is believed to cause insulin resistance (Wajchenberg 2000, Masuzaki *et al.* 2001). In obesity, adipocytes are enlarged and increased in number, and an excess of lipid leads to ectopic deposition of triglyceride in the liver and muscle, which is one of the causes of insulin resistance (Savage *et al.* 2007). The hypertrophied adipocytes produce proinflammatory cytokines such as monocyte chemoattractant protein-1 (MCP1 (CCL2)) and tumor necrosis factor- α (TNF- α) as obesity progresses (Shoelson *et al.* 2006). These cytokines, so-called adipokines, cause inflammation and recruitment of macrophages to adipose tissue (Xu *et al.* 2003), which is another important mechanism for obesity-induced insulin resistance. The infiltrated macrophages enhance inflammation of adipose tissue, indicating that these processes form a vicious circle.

TNF- α induces c-Jun amino-terminal kinase (JNK) activation and phosphorylation of insulin receptor substrate 1 (IRS1) at serine residues that negatively regulate normal signaling through the insulin receptor/IRS1 axis (Hotamisligil *et al.* 1996). Mice lacking chemokine receptor-2 (CCR2), a receptor for MCP1, are partly protected against developing high-fat diet (HFD)-induced insulin resistance and exhibit reductions in adipose tissue macrophage recruitment and inflammatory gene expression (Weisberg *et al.* 2006). These observations suggest that adipose tissue in obesity is characterized by chronic low-grade inflammation, and inflammatory cytokines play a causative role in the development of insulin resistance.

Beraprost sodium (BPS) is a stable prostaglandin I₂ analogue and has a potent vasodilating effect through activation of prostacyclin receptor (Olschewski *et al.* 2004). BPS is also reported to attenuate inflammation. BPS reduced serum TNF- α levels in diabetic patients (Fujiwara *et al.* 2004) and expression of *Mcp1* mRNA in the kidney of Otsuka Long-Evans Tokushima fatty (OLETF) rats, resulting in the amelioration of diabetic nephropathy (Watanabe *et al.* 2009). BPS inhibits

TNF- α -induced expression of vascular cell adhesion molecule and monocyte attachment to endothelial cells (Goya *et al.* 2003). We therefore hypothesized that the anti-inflammatory effects of BPS may be beneficial for the improvement of obesity-induced insulin resistance, in which inflammation plays an important role.

We showed in this study that BPS improved glucose metabolism in association with reduction of inflammation of white adipose tissue (WAT) in a mouse model of diet-induced obesity.

Materials and Methods

Animals

All procedures were approved by the institutional animal use and care committee and were conducted in conformity with institutional guidelines. Four-week-old C57/B6 mice were purchased from Kyudo Co. Ltd. (Tosu, Saga, Japan). Three groups were analyzed: mice fed a normal chow diet (control group), mice fed a HFD containing 60% kcal fat (High Fat Diet 32, Clea Japan (Tokyo); HFD group) for 12 weeks, and mice fed a HFD and administered BPS for 12 weeks (BPS group). BPS dissolved in water at 1.5 μ g/ml was given *ad libitum* because of the short half-life of BPS ($\sim$ 1 h). As a preliminary study showed that the estimated volume of water intake was $\sim$ 0.2 ml/g per day, the estimated dose of orally ingested BPS was 300 μ g/kg per day. At the end of the experiment, systolic blood pressure (SBP) and heart rate (HR) were measured by a tail-cuff method (BP-98A, Softron Co., Tokyo, Japan). Mice were killed by CO₂ inhalation.

Histological analysis

Adipose tissues were fixed with 10% formaldehyde for 24 h. The specimens were embedded into paraffin. Paraffin sections were stained with hematoxylin and eosin (H&E). The cross-sectional area for each adipocyte was determined using Dynamic cell count BZ-HIC (Keyence, Osaka, Japan). To detect macrophage infiltration, the paraffin sections were stained with an antimouse MAC3 (LAMP2) antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Sections were deparaffinized with xylene and refixed with ethanol for 40 min, immersed in PBS, and then autoclaved in citrate buffer for antigen retrieval. Then, the sections were incubated with 3% hydrogen peroxide in methanol for 20 min. The sections were further incubated with an antibody against MAC3 (1:200) overnight at 4 °C. After rinsing with PBS, the sections were incubated with biotin-labeled goat anti-rabbit IgG antiserum (Santa Cruz Biotechnology, Inc., 1:200 dilution) for 30 min and then incubated with avidin–biotin complex (Vectastain ABC kit; 1:100 dilution) for 15 min, and the sections were incubated with 3,3'-diaminobenzidine and 0.03% hydrogen peroxide in deionized water for about 80 s. The number of MAC3-positive cell clusters was counted in high power field (HPF). The data are mean of five randomly chosen HPFs. After the mice were killed, the livers were removed and subsequently fixed in 10% formaldehyde. The sections were embedded in

paraffin blocks and stained with H&E to examine the structures of the liver and evaluate lipid droplets. For the quantification of areas of lipid accumulation in the liver, H&E-stained images of liver were uploaded into a computer for analysis. The images were processed into two gradations (black and white). The white porosity areas were quantified as vacuolation (Sato *et al.* 2010), which mostly represents accumulation of lipid droplets. Contribution of arteries, veins, and bile ducts to porosity area was small and equally observed in the three groups and therefore ignored in the quantification. The data are expressed as a percentage of white area to total area.

Glucose tolerance test and insulin tolerance test

Mice were starved for 6 h. Then, the mice were i.p. injected with glucose (1 g/kg of body weight) for the glucose tolerance test (GTT). For the insulin tolerance test (ITT), the mice were i.p. injected with rapid insulin (0.5 IU/kg of body weight). Blood was taken from tail vein at various time points to measure blood glucose concentrations by Glutest Every (Sanwa Kagaku Kenkyusho, Nagoya, Japan).

Measurement of serum levels of triglyceride, cholesterol, and insulin

Serum triglyceride and total cholesterol levels were determined by commercially available kits, Triglyceride E-test Wako (Wako, Osaka, Japan) and Cholesterol E-test Wako (Wako) respectively. Serum insulin levels were determined by ELISA kit (Morinaga Institute of Biological Science, Yokohama, Japan).

RNA extraction and real-time quantitative RT-PCR analysis

RNA from adipose tissue was extracted using ISOGEN according to the manufacturer's instruction (Wako). One microgram of total RNA was reverse transcribed using ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan). Real-time quantitative PCR (qPCR) was performed using 7500 real-time PCR system (Applied Biosystems) and SYBER Green PCR Master Mix (Applied Biosystems). Primer sequences for real-time qPCR used in this study are as follows:

TNF- α : 5'-AAGCCTGTAGCCCACGTCGTA-3';
5'-GGCACCAGTGTGTTGTCTTTG-3'.
MCP1: 5'-TTAACGCCCCACTCACCTGCTG-3';
5'-GCTTCTTTGGGACACCTGCTGC-3'.
PPAR γ (PPARG):
5'-TGTCGGTTTCAGAAGTGCCTTG-3';
5'-TTCAGCTGGTCGATATCACTGGAG-3'.
C/EBP α : 5'-TTGAAGCACAAATCGATCCATCC-3';
5'-GCACACTGCCATTGCACAAG-3'.
Adiponectin: 5'-GTCAGTGGATCTGACGACACCAA-3';
5'-ATGCCTGCCATCCAACCTG-3'.
18S rRNA: 5'-ACTCAACACGGGAAACCTCA-3';
5'-AACCAGACAAATCGCTCCAC-3'.

The expression of each gene was normalized with that of 18s rRNA.

ELISA

ELISA for adiponectin and TNF- α was performed using commercially available kits (R&D systems, Inc., Minneapolis, MN, USA) in accordance with the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed with one-way ANOVA and Fisher's test if appropriate. Data are shown as mean $\pm$ S.E.M. $P < 0.05$ was considered to be statistically significant.

Results

Hemodynamic and metabolic parameters

Body weight was significantly increased in the HFD group compared with the control group at the end of the experiment. However, there was no significant difference between the HFD and BPS groups (Table 1). SBP was significantly increased in the HFD group compared with the control group. BPS significantly reduced SBP, but SBP in the BPS group was still significantly higher than that in control group (Table 1). HR was significantly increased in the HFD group compared with the control group. BPS reduced HR, but the difference was not statistically significant (Table 1).

After 16 h of fasting, lipid profile and glucose and insulin levels were determined. Total cholesterol and triglyceride were significantly increased by the HFD (Table 2). BPS treatment modestly decreased total cholesterol and triglyceride levels. However, the differences were not statistically significant. Fasting insulin and glucose levels were elevated in the HFD group.

BPS improved GTT and ITT

At the end of experiment, GTTs and ITTs were performed. The HFD group developed glucose intolerance (Fig. 1A) and insulin resistance (Fig. 1C). Treatment of mice with BPS significantly improved glucose tolerance and insulin action (Fig. 1A and C). Interestingly, the basal glucose level was

Table 2 Fasting serum chemistry of control, HFD-, and BPS-treated mice

	TC (mg/ml)	TG (mg/ml)	Insulin (ng/ml)	Glucose (mg/ml)
Control (n=5)	55 $\pm$ 4	66 $\pm$ 12	0.16 $\pm$ 0.05	59.6 $\pm$ 2.6
HFD (n=10)	209 $\pm$ 9 [†]	94 $\pm$ 9*	3.75 $\pm$ 0.51 [†]	142.4 $\pm$ 7.3 [†]
HFD+BPS (n=10)	182 $\pm$ 14 [†]	74 $\pm$ 6	4.12 $\pm$ 0.90 [†]	132.8 $\pm$ 6.2 [†]

HFD, high-fat diet; BPS, beraprost sodium; TC, total cholesterol; TG, triglyceride. * $P < 0.05$ and [†] $P < 0.01$ vs control.

significantly lower in the BPS group compared with the HFD group after 6 h of fasting, which was not observed after 16 h of fasting (Table 2). Area under the curves (AUC) also showed improvement of glucose metabolism by BPS treatment (Fig. 1B and D).

BPS reduced adipocyte size

Histological analysis of epididymal WAT showed that the adipocyte size was increased in the HFD group compared with the control group (Fig. 2A and B). Treatment with BPS reduced adipocyte size (Fig. 2C). Statistical analysis confirmed that BPS significantly reduced adipocyte size (Fig. 2D). These data suggest that BPS enhanced adipocyte differentiation. We therefore examined expression of genes related to adipocyte differentiation. *Ppar γ* was significantly suppressed by the HFD, which was reversed by BPS (Fig. 3A). The upregulation of *Ppar γ* by BPS showed a borderline significance ($P = 0.06$) when three groups were considered. However, the difference between the HFD and BPS groups was statistically significant if only the HFD groups were compared ($P = 0.02$). We failed to see a significant effect of BPS treatment on the expression of C/EBP α (Fig. 3B) or adiponectin (Fig. 3C). Although adiponectin mRNA levels were not changed in the three groups, serum adiponectin levels were mildly decreased in the HFD group and BPS groups (Fig. 3D). However, the difference in serum adiponectin levels between the three groups was not statistically significant.

BPS reduced inflammatory changes in WAT in HFD-fed mice

Chronic inflammation in WAT is a common feature of obesity. Therefore, we examined the infiltration of macrophages into adipose tissue. The number of MAC3-positive macrophage aggregation surrounding adipocytes, often referred to as a crown-like structure (CLS) in WAT (Weisberg *et al.* 2003, Xu *et al.* 2003), was significantly increased in the HFD group compared with the control group (Fig. 2E and F; arrowheads). In the control group (Fig. 2E), almost no CLS was observed in WAT. Treatment with BPS significantly decreased the number of CLSs in WAT (Fig. 2G and H).

Table 1 BW, SBP, and HR

	BW (g)	SBP (mmHg)	HR (b.p.m.)
Control (n=5)	26.9 $\pm$ 0.5	97 $\pm$ 3	448 $\pm$ 31
HFD (n=10)	43.4 $\pm$ 1.1 [†]	114 $\pm$ 2 [†]	532 $\pm$ 15 [†]
HFD+BPS (n=10)	43.9 $\pm$ 0.9 [†]	106 $\pm$ 2* [†]	496 $\pm$ 15

HFD, high-fat diet; BPS, beraprost sodium; BW, body weight; SBP, systolic blood pressure; HR, heart rate. * $P < 0.05$, [†] $P < 0.01$ vs control. [‡] $P < 0.05$ vs HFD group.

Figure 1 BPS ameliorated HFD-induced insulin resistance. (A) The control group (black triangle) was fed a normal chow and the HFD (black box) and BPS groups (white box) were fed a HFD for 12 weeks. Mice were i.p. injected with 1 g/kg glucose and blood glucose levels were measured. (C) Mice were i.p. injected with 0.5 U/kg insulin and blood glucose levels were measured. (B and D) AUC were calculated. Control group $n=5$, HFD group and BPS group $n=10$. * $P<0.05$ and ** $P<0.01$ vs control. # $P<0.05$ vs HFD group, ## $P<0.01$ vs HFD group.

Real-time PCR analysis showed that HFD-induced expression of $TNF-\alpha$ and MCP1 was significantly suppressed by BPS treatment (Fig. 3E and F). We could not detect serum $TNF-\alpha$ even in mice fed a HFD (data not shown).

BPS attenuated HFD-induced hepatic steatosis

Finally, we examined whether BPS affects hepatic steatosis induced by the HFD. HFD feeding for 12 weeks caused fatty liver compared with control feeding (Fig. 4A and B). Fat accumulation in the liver was attenuated in the BPS group compared with the HFD group (Fig. 4C and D).

Discussion

We demonstrated in this study that BPS improved HFD-induced insulin resistance and glucose intolerance. Treatment with BPS reduced expression of inflammatory cytokines, adipocyte size, and macrophage infiltration in WAT of diet-induced obesity mice. BPS also induced modest $PPAR\gamma$ upregulation. Although BPS treatment did not affect serum glucose and insulin levels after 16 h of fasting (Table 2), GTT performed after 6 h of fasting showed a significant reduction in basal glucose levels in the BPS group. The difference may be ascribed to the length of the fasting period, and prolonged fasting time may attenuate the difference between the HFD group and the BPS group.

Low-grade adipose tissue inflammation is a key state underlying insulin resistance in obesity. An increase in $TNF-\alpha$ mRNA expression was observed in adipose tissue from animal models of obesity and diabetes (Hotamisligil *et al.* 1993). It has

been suggested that $TNF-\alpha$ is an important mediator of insulin resistance in obesity because neutralization of $TNF-\alpha$ increased peripheral glucose uptake in response to insulin in obese rats. And $TNF-\alpha$ -deficient obese mice were protected against obesity-induced attenuation of insulin signaling in muscle and fat tissues (Uysal *et al.* 1997). Several studies suggest that $TNF-\alpha$ blocks insulin signaling. It is reported that $TNF-\alpha$ inhibits insulin-induced tyrosine phosphorylation and tyrosine kinase activity of the insulin receptor in the obese rat (Hotamisligil *et al.* 1994a,b). $TNF-\alpha$ activates JNK signaling, and JNK activation promotes the

Figure 2 BPS treatment decreased adipocyte size and infiltration of macrophages in WAT. (A, B, and C) Representative microphotographs of H&E-stained sections of epididymal WAT from the control group (A), HFD group (B), and BPS group (C) are shown. Scale bar, 100 μm . (D) Bar graph indicates average adipocyte size of epididymal WAT, $n=5$ (control group; $n=3$). ** $P<0.01$ vs control. ## $P<0.01$ vs HFD group. (E, F, and G) Representative microphotographs of WAT immunohistochemically stained with an anti-MAC3 antibody to stain macrophage in the control group (E), HFD group (F), and BPS group (G) are shown. Scale bar, 100 μm . (H) Bar graph indicates the number of CLSs in high power field (HPF), $n=7-8$. ** $P<0.01$ vs HFD group. Almost no CLS was observed in the control group.

Figure 3 Effect of BPS on the adipocyte differentiation markers in WAT. (A, B and C) The results of real-time qPCR analysis for PPAR γ (A), C/EBP α (B), and adiponectin (C) are shown, $n=5$. ** $P<0.01$ vs control. (D) Serum adiponectin levels were determined by ELISA. (E and F) The results of real-time qPCR analysis for TNF- α (E) and MCP1 (F) expression in WAT are shown, $n=5$. ** $P<0.01$ vs control. # $P<0.05$ vs HFD group.

phosphorylation of IRS1 at serine residues that negatively regulate normal signaling through IRS1 (Aguirre *et al.* 2000, Shoelson *et al.* 2006). An absence of JNK1 (MAPK8) improved insulin sensitivity and enhanced insulin receptor signaling in diet-induced obesity mice and *ob/ob* genetic obesity mice (Hotamisligil *et al.* 1993, Hirosumi *et al.* 2002). In this study, BPS reduced mRNA expression of TNF- α in WAT from diet-induced obesity mice. Hence, we assume that reduction of TNF- α may contribute to the improvement of insulin action by BPS, at least in part. It is also expected that the signaling pathways activated by TNF- α are attenuated in the adipose tissue of BPS-treated mice. However, the effect of BPS seems to be multi-fold, such as reduction of MCP1. Therefore, it is difficult to determine specifically whether TNF- α -signaling is attenuated in this model. It is important to note that we must be cautious in extrapolating the data in this study to humans because TNF- α neutralizing antibodies have been shown to be ineffective on impacting insulin sensitivity in humans (Ofei *et al.* 1996).

Several studies showed anti-inflammatory effects of BPS. Ohta *et al.* (2005) showed that BPS suppressed concanavalin-A-induced TNF- α and INF- γ production and liver injury. It is also reported that BPS prevented the development of cigarette smoke extract-induced emphysema (Chen *et al.* 2009). Production of TNF- α and interleukin 1 β (IL β) in the lung

tissue was suppressed by BPS in this model. Although activation of prostacyclin receptor is suggested to be involved in the anti-inflammatory effect, the detailed mechanism of BPS suppression of inflammatory cytokine production is not clear and further study is needed.

Hypertrophied adipose tissue secretes MCP1 (Hotamisligil *et al.* 1995, Bruun *et al.* 2005). MCP1 attracts macrophages to adipose tissue (Kamei *et al.* 2006). It is suggested that infiltrated macrophages secrete MCP1 and proinflammatory cytokines such as TNF- α or IL6, indicating that macrophage infiltration and inflammation of WAT form a vicious circle (Suganami *et al.* 2005). It is also reported that transgenic overexpression of MCP1 in adipose tissue causes insulin resistance by direct attenuation of insulin signaling in skeletal muscle and liver and inflammation of adipose tissue (Kamei *et al.* 2006, Kanda *et al.* 2006). Therefore, MCP1 is thought to contribute to insulin resistance through paracrine and endocrine effects (Kamei *et al.* 2006). BPS reduced both MCP1 expression and macrophage infiltration, which may contribute to the improvement of glucose tolerance and insulin action.

PPAR γ has been known as a master regulator of adipocyte differentiation *in vivo* and *in vitro* (Rosen *et al.* 1999, Camp *et al.* 2002). PPAR γ ligands, such as thiazolidinediones, improve insulin sensitivity by increasing the number of small adipocytes secreting adiponectin and decrease the number of large adipocytes secreting TNF- α in WAT (Okuno *et al.* 1998, Yamauchi *et al.* 2001). We observed that treatment with BPS decreased the average size of adipocytes in epididymal WAT from diet-induced obese mice. Although statistical significance was marginal, it is plausible to assume that the increased PPAR γ by BPS may contribute to adipocyte

Figure 4 BPS attenuated HFD-induced hepatic steatosis. (A, B, and C) Representative microphotographs of H&E-stained sections of liver from the control group (A), HFD group (B), and BPS group (C) are shown. Scale bar, 500 μ m. (D) Bar graph indicates area of vacuolation, $n=5-8$. ** $P<0.01$ vs control. ## $P<0.01$ vs HFD group.

differentiation and improvement of insulin action. Because PPAR γ is known to inhibit NF- κ B, a transcription factor-mediated expression of inflammatory cytokines, BPS may suppress TNF- α and MCP1 expression through PPAR γ activation. Although the detailed mechanism of BPS upregulation of PPAR γ is not clear, a previous report showed that another prostacyclin analogue, treprostinil, activates PPAR γ through prostacyclin receptor-dependent and cAMP-independent mechanisms (Falcetti *et al.* 2007).

Adipocytes become hypertrophied as obesity progresses. It is suggested that these hypertrophied adipocytes secrete free fatty acid that contributes to fat accumulation in ectopic sites including liver and muscle, leading to the development of insulin resistance (Jacob *et al.* 1999, Bays *et al.* 2004, Hwang *et al.* 2007). Although the mechanism is not clear, BPS mildly decreased serum TC and TG levels (Table 2). A decrease in the serum TC and TG levels by BPS may contribute to the improvement of insulin action.

It is of note that BPS ameliorated hepatic steatosis induced by HFD. Although the precise mechanism is not clear, anti-inflammatory effects of BPS may play a role in the attenuation of hepatic steatosis because recent studies suggest that inflammation is one of the critical factors in the development of hepatic steatosis. It was reported that anti-TNF α antibody treatment improved hepatic steatosis in *ob/ob* mice (Li *et al.* 2003). Treatment with an inhibitor of MCP1 receptor reportedly attenuated insulin resistance and hepatic steatosis in diet-induced obese mice (Tamura *et al.* 2010). Therefore, reduction of fat accumulation in the liver by BPS treatment may be due to suppression of inflammatory cytokine expression, at least in part, as observed in WAT of the BPS group. However, it is not clear at this point whether cytokine levels in the liver are decreased in the BPS group, and further study is needed.

A recent study showed that BPS improves glucose metabolism in a genetic obesity-induced insulin resistance model (Sato *et al.* 2010). Although our data mostly agree with those by Sato *et al.* (2010), our study is also important because we showed beneficial effects of BPS on glucose metabolism in a diet-induced obesity model, which is more clinically relevant than a genetic model. In addition, our data suggest a possible involvement of anti-inflammatory effects of BPS in the improvement of impaired glucose metabolism, which was not obvious in the previous study by Sato *et al.* (2010).

Taken together, we showed in this study that BPS improved glucose tolerance in mice fed a HFD possibly through suppression of inflammatory cytokines in WAT and induction of adipocyte differentiation. BPS may be beneficial not only for the treatment of patients with peripheral artery disease or pulmonary hypertension but also for treatment of patients with insulin resistance.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

E I researched the data, contributed to discussion, wrote and reviewed/edited the manuscript. T I contributed to discussion and wrote and reviewed/edited the manuscript. K T and K S reviewed/edited the manuscript. H M, T H, J I, and A K researched the data.

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