Fenofibrate Reverses Palmitate Induced Impairment in Glucose Uptake in Skeletal Muscle Cells by Preventing Cytosolic Ceramide Accumulation

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Key Words
Carnitine palmitoyl transferase • Fatty acid oxidation • High-fat diet • Lipotoxicity • Obesity • PPAR-agonist • Insulin resistance • Palmitic acid

Abstract
Backgrounds/Aims: The lipid induced insulin resistance is a major pathophysiologic mechanism underlying glucose intolerance of varying severity. PPAR\textsubscript{\alpha}-agonists are proven as effective hypolipidemic agents. The aim of this study was to see if impaired glucose uptake in palmitate treated myotubes is reversed by fenofibrate. Methods: Palmitate-treated myotubes were used as a model for insulin resistance, impaired glucose uptake, fatty acid oxidation and ceramide synthesis. mRNA levels of CPT1 and CPT2 were determined by PCR array and Q-PCR. Results: The incubation of myotubes with 750 \textmu M palmitate not only reduced glucose uptake but also impaired fatty acid oxidation and cytosolic ceramide accumulation. Palmitate upregulated CPT1\textsubscript{b} expression in L6 myotubes, while CPT2 expression level remained unchanged. The altered stoichiometric ratio between the two CPT isoforms led to reduced fatty acid oxidation (FAO), ceramide accumulation and impaired glucose uptake, whereas administration of 200 \textmu M fenofibrate significantly reversed the above abnormalities by increasing CPT2 mRNA levels and restoring CPT1\textsubscript{b} to CPT2 ratio. Conclusion: Palmitate-induced alteration in the stoichiometric ratio of mitochondrial CPT isoforms leads to incomplete FAO and enhanced cytosolic ceramide accumulation that lead to insulin resistance. Fenofibrate ameliorated insulin resistance by restoring the altered stoichiometry by upregulating CPT2 and preventing, cytoplasmic ceramide accumulation.
Introduction

The term ‘peripheral insulin resistance’ refers to suboptimal response of insulin target tissues to the metabolic effects of insulin and often links to metabolic disorders like diabetes and obesity. Abnormal storage of neutral lipids in the liver, adipose tissue and skeletal muscle makes them dysfunctional in terms of their metabolic capacity [1-3]. However, genetic manipulations that increase tissue levels of these lipid molecules do not always lead to insulin resistance [4]. It has been suggested that obesity-associated glucose intolerance may in fact result from the defective handling of metabolic fuels by the mitochondria [5, 6].

Skeletal muscle cells, quantitatively the most important site for peripheral glucose uptake, lose some of their capacity to absorb and utilize glucose, when loaded with FFA [7-9]. Elevated plasma FFA levels interfere with glucose uptake by myocytes, leading to the development of metabolic syndrome and type 2 diabetes (T2D) [10, 11]. FFAs enhance ROS production due to increased mitochondrial uncoupling and FAO [12-16]. The hypothesis suggesting oxidative stress as a causative factor for the development of insulin resistance have been supported by several studies that showed improvement in insulin resistance following reversal of the imbalance between ROS and antioxidants [17-19]. Apart from becoming apoptotic, cells loaded with FFA also show impairment in FAO and glucose uptake. Excess FFA leads to ‘lipotoxicity’ by the intracellular accumulation of triglyceride (TG), ceramides and other fatty acid derivatives that impair mitochondrial β-oxidation and insulin resistance in skeletal muscle [20-23]. Recent studies have shown modulation of cytosolic ceramides through altered ceramide synthase expression improves insulin sensitivity [24]. Palmitate (PA) plays a significant role in regulating intracellular ceramide levels by inducing serine palmitoyl transferase (SPT) and also by causing hydrolysis of sphingomyelins. Cytosolic ceramides inhibit insulin signaling, although some isoforms have been shown to induce insulin signaling [25].

β-oxidation of fatty acyl-CoA ester occurs in the mitochondria of myotubes under the influence of CPT isoforms. Fatty acyl-CoA that escapes β-oxidation is stored as TG. CPT1 in the outer mitochondrial membrane that transports fatty acyl-carnitine from cytosol to intermembrane space. CPT1b is the major isoform expressed in muscle and is rate-limiting for FAO. Upregulation of Cpt1 is associated with excess FA uptake into the cell. CPT2 in the inner mitochondrial membrane transports fatty acyl-carnitine to the mitochondrial matrix for β-oxidation. Since the activity of both CPT1 and CPT2 is essential for the transport of FA into mitochondria, altered expression of one or both of these isoforms impairs FAO. Peroxisome proliferator activated receptor (PPAR) α, β and γ belong to a family of nuclear hormone receptors. The receptors are bound and activated by FFAs and they regulate a number of genes involved in lipid metabolism. Studies using PPARα null mice suggest a critical role for this receptor in maintaining the constitutive activity of β-oxidative pathways in liver and heart [26]. PPARα-agonists are proven as effective hypolipidemic agents, and new evidence indicate that they might also prove effective for treating obesity and insulin resistance [27]. The current study was undertaken to investigate the role of CPT isoforms in palmitate-induced insulin resistance in skeletal muscle cells and to explore the possibilities to restore this by therapeutic interventions.

Materials and Methods

Cell culture

Mouse and rat skeletal muscle cell lines, C2C12 and L6 myoblasts, were obtained from the National Centre for Cell Science, Pune, India. Cells were maintained in DMEM (Gibco™ Invitrogen, USA) supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco™) at 37 °C in 5% CO₂ and grown on six-well plates (BD Biosciences) at a density of 3x10⁴/well in 3 ml of growth medium. 3d after plating, the cells reached ~80-90% confluence (day 0), then differentiated by replacing the growth medium with DMEM
(4.5 g/l glucose) supplemented with 2% FCS, 1nM insulin, 100 U/ml penicillin and 100 μg/ml streptomycin (differentiation medium). This medium was changed every 24 h, and the differentiated cells (at days 4 and 5) were used for subsequent experiments. FFA-containing media were prepared by pre-incubation of FFA (Sigma-Aldrich, USA) with DMEM and 2% BSA (fraction V) [28]. A stock of 100 mM of different FAs (Sigma-Aldrich) were prepared in 0.1 (N) NaOH by heating at 70°C. FAs were conjugated with FA-free BSA (fraction V, Sigma) using vortex to make 2 mM working stock. This mixture was then filtered and sterilized prior to addition to the media. 750 uM of PA (final conc) were used for treatment in cell culture unless otherwise specified.

Glucose uptake assay
Glucose uptake assay in muscle cells was performed as described earlier [29, 30]. FA-pretreated myotubes were incubated in serum-free DMEM in presence or absence of 100 nM insulin for 15m at 37°C in a 24 well plate. Cells were then treated with 0.5 ml 10 µM 2-deoxy-D-[H-3] glucose [1 μCi/μl] for 10m. The reaction was terminated with two rapid washes of ice-cold PBS buffer containing 20 µM phloretin (Sigma). The cells were lysed with 1% NP-40 and the radioactivity was determined by liquid scintillation counting (Packard, TRI-CARB 2100TR). Lowry method was used to determine the protein concentration of each well to determine the final radioactivity per unit protein.

Fatty acid oxidation (FAO) assay
Mitochondrial FAO rate in muscle cells was determined as described earlier [31]. L6 and C2C12 cells were cultured in 12-well dishes, as described above. Differentiated and fatty acid pre-treated cells were incubated for an additional 3h with fresh DMEM, 1% FBS that was supplemented with 3.0 μCi/μl [1-14C] palmitic acid (Amersham Pharmacia Biotech, UK). The oxidation reactions were terminated and CO2 released from the media by the addition of 70% perchloric acid was captured on filter paper saturated with 3M NaOH. Following 3h of incubation with gentle shaking at 25°C, 14CO2 resulting from oxidized fatty acid was quantified by scintillation counting (Packard). Each experiment was performed in triplicate and the results were represented as per protein content.

Localization of radioactivity of 14C-palmitic acid in cell
L6 cells were differentiated and co-treated with 750 µM palmitic acid supplemented with [1-14C] palmitic acid (Amersham). After 18h incubation, the cells were harvested to determine the total cellular radioactivity. Mitochondria were then isolated from the cells by differential centrifugation and radioactivity/mg of mitochondrial protein was separately measured. Percentage of mitochondrial radioactivity was expressed as the ratio of mitochondrial to total cellular radioactivity.

Radiolabeling of lipids and determination of total cellular ceramide
Ceramide was analyzed as described earlier [32]. Cellular lipids were radio-labeled by adding [1-14C] palmitic acid (Amersham, 2.5 μCi/ml) to the culture medium for 24h. Radiolabeled ceramide was isolated by lipid extraction and thin layer chromatography (TLC). Total lipids were extracted from cells using chloroform-methanol (2:1) solvent and separated by TLC using chloroform-acetic acid (9:1) resolving media. After drying, the lipids were visualized by iodine vapor and the position of ceramide was determined by comparison with a standard C-16 ceramide (Avanti Polar Lipids), which were co-migrated with total lipids. Designated spots of ceramides were scraped off and quantified by liquid scintillation (Packard).

RNA isolation and cDNA preparation
Total RNA was isolated from the cells using the RNeasy Mini Kit (Qiagen, CA). The cDNA synthesis was carried out using M-MuLV Reverse transcriptase (Fermentas, USA). To the reaction mix, 1µl oligodT(-18) primer was added to 500 ng of total RNA and the volume was made up to 12 µl with RNase-free water, gently mixed and incubated for 5 min at 70°C and then chilled on ice. To this mixture 4µl of 5x RT reaction buffer, 1µl of RiboLock™ RNase inhibitor (20 U/µl, Fermentas) and 2µl of 10 mM dNTP mix was added and then incubated at 37°C for 5 min. Finally, 1µl of M-MuLV RT (200 u/µl) was added and the reaction was carried out at 42°C for 1h and was stopped after incubation at 70°C for 10 min. The cDNA mixture was then chilled on ice and stored at ambient temperature until subsequent PCR reactions.
Mitochondrial PCR-array

Each RNA sample (0.5 µg) was mixed with 2 µl of genomic DNA Elimination Buffer and made up to 10 µl, incubated at 42°C for 5 minutes and chilled on ice for 1 min. To this mixture, 10 µl of RT cocktail (prepared according to the manufacturer’s instruction) was added and incubated at 42°C for 15 min, followed by heating at 95°C for 5 min. 91 µl of ddH$_2$O was added to each 20 µl of cDNA Synthesis Reaction. Q-PCR was then performed using the Rat Mitochondria RT² Profiler™ PCR Array according to the manufacturer’s protocol (QIAGEN-Super-Array Bioscience, Frederick, MD). Gene expression was compared according to the C$_T$ value. The normalizer used for each cDNA sample was the average of five housekeeping genes, viz., Hypoxanthine phosphoribosyltransferase 1, β-2-microglobulin, Ribosomal protein L13a, Glyceraldehyde-3-phosphate dehydrogenase and β-actin.

Real time quantitative PCR and CT value calculation

Real time PCR was carried out by ABI 7500 Real Time PCR System with Power SYBR® Green (ABI) as described previously [33]. The 20µl reaction mixture contained 10µl of 2X master mix, 1µl each of forward and reverse primers (10pmol), 2µl cDNA and 6µl DNase-free water. 18s rRNA and actin were used as an endogenous control for each gene. The oligonucleotide primers used as in the reactions have been listed in Table 1.

In Q-PCR, relative quantification was performed by comparative C$_T$ value calculation. In this method arithmetic formulae are used to calculate relative expression levels, compared with a calibrator, which can for instance be a control (non-treated) sample. The amount of target normalized to an endogenous housekeeping gene and relative to the calibrator, is then given by $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T = \Delta C_T$ (sample) - $\Delta C_T$ (calibrator), and $\Delta C_T$ is the C$_T$ of target gene subtracted from the C$_T$ of the housekeeping gene [32].

Glut-4 cell membrane translocation assay

Glut-4 translocation from cytosol to the plasma membrane was determined by FACS analysis as described previously [34, 35]. Briefly, after experimental treatment, cells were washed with PBS, and incubated for 30 min with 7.5 µl Glut-4 antibody (Santa Cruz, cat. sc-53566) that was earlier conjugated with 1.5 µl Alexa flour-488 secondary antibody for 10m. After the incubation, cells were washed with PBS and then incubated in PBS containing 1% PFA for 10m. Then the cells were harvested and washed twice with PBS. Finally, cells were resuspended in PBS containing 1% PFA and were analyzed by FACS.

Statistical analysis

All results were expressed as means ± SE. Statistical calculations were performed using two-way ANOVA, followed by Duncan’s post hoc test (p <0.05) for comparison of multiple groups. Data analysis was performed using the computer program Microcal origin version 6.0 software. The experiments were repeated at 3-times unless otherwise stated. To make the variance independent of the mean, statistical analysis of quantitative RT-PCR data is presented after logarithmic transformation.

Results

FFAs show differential impact on Insulin-mediated glucose uptake in muscle cells

While incubation of L6 myotubes with different concentrations of palmitoleic acid, linoleic acid, and arachidonic had no significant inhibitory effect, Palmitic Acid significantly
Fig. 1. (a) The Effect of different FFAs (750 µM each) on glucose uptake in skeletal muscle cells. The myotubes were treated with different FFAs as mentioned below each bar in presence or absence of insulin (100 nM for 30m) and [1H]-2-deoxyglucose (1 μCi/μl) containing KRP buffer for 10m. (b) The effect of 750 µM PA on glucose uptake in presence of insulin by differentiated myotubes at different time points has been shown. (c-f) The effect of FFAs at different doses on glucose uptake in skeletal muscle cells. The statistical analysis is done as described in Methods section.* represents (p < 0.05).
reduced insulin stimulated glucose uptake after 18h of incubation. (Fig. 1a, c-f). Glucose uptake was maximally suppressed 18h after PA treatment, no further suppression occurred with time (Fig. 1b). Henceforth, we selected PA-treatment for 18h in subsequent experiments.

Excess PA inhibits self-entry into the mitochondria resulting in reduced rate of FAO

Entry of radio labeled $^{14}$C-PA in mitochondria of differentiated L6 myotubes was determined in the presence or absence of excess PA. Mitochondrial and total cellular radioactive intermediates were measured separately in each case. While 80% of the radio labelled PA was taken up by the mitochondria in the control myotubes, only 50% radioactivity was detected following PA loading (Fig. 2b). Thus, PA-excess interfered with its own uptake by the mitochondria, leading to cytosolic accumulation. FAO rate was reduced by 40% following treatment with 750 µM PA for 18h (Fig. 2a).

Time kinetics study of $^{14}$C-PA incorporation into mitochondria and cytosol shows localization of palmitic acid in these two compartments upon PA-treatment. PA-treated whole cell as well as isolated mitochondria imports $^{14}$C-PA at a higher rate than that of
control till 8h but a significant fall in this import is observed beyond 8h (Fig. 2c II). Moreover, the count in cytosol gradually increased with time up to 18h in PA-treatment cells (Fig. 2c III) whereas the radioactive count decreased in mitochondrial fraction in PA-treated cells (Fig. 2c II) indicating accumulation of \(^{14}\)C-PA derivatives in the cytosol.

Ceramide overproduction due to excess cytosolic PA was reversed by fibrates
Production of C-16 ceramide was determined using \(^{14}\)C-PA in the excessive PA-treated and in fibrate treated cells. Increase in PA concentration caused excess ceramide production; 750 µM PA produced about three times more ceramide than control. This PA-induced ceramide production was abolished by Fenofibrate and Bezafibrate treatment to myotubes (Fig. 3).

Fig. 3. The ceramide production in myotubes following treatment of PA and agents like fenofibrate and bezafibrate. Cells were treated with radioactive PA. After isolation of radioactive ceramide by TLC technique, radioactivity was measured per mg protein. Significant increase of ceramide after 750 µM PA-incubation was almost normalized by fenofibrate and bezafibrate. The statistical analysis is done as described in Materials and Methods. * represents \((p < 0.05)\).
PA-treatment differentially regulates genes associated with mitochondrial function in muscle cell

To determine the effect of 750 μM PA treatment for 18h on gene expression in differentiated rat skeletal muscle cells (L6), expression of 84 mitochondria-associated genes were analyzed using PCR array as mentioned in the method section. These are nuclear genes associated with mitochondrial function. Among 84 genes (Fig. 4), we found significant (>2 fold) up regulation of 30 genes associated with mitochondrial fission/fusion, membrane polarization/potential as well as membrane transport. Some of the genes are solute carrier (Slc) family genes, mitochondrial transport related genes like Bcl2l1, Cpt1b, Cpt2, mitochondrial fission/fusion controlling genes like Fis1, Mfn1, membrane polarization/potential regulating genes, like Bcl2, Bcl2l1, Tf53, Ucp2, Ucp3 and apoptotic genes, like Akt1, Bbc3, Bcl2l1, Sfn and Tf53. The results suggest that these genes are significantly (4-8 fold) increased or decreased upon PA-treatment (Fig. 4).
PA-treatment in cells alters the ratio of CPT1b and CPT2, which is partially restored by fibrates

From the data obtained out of Mitochondrial RT² Profiler PCR array screening, Cpt1b and Cpt2 differential regulation was observed in PA-treated myotubes. We further validated the expression profile by Q-PCR that showed upregulation of Cpt1b upon PA-treatment and the expression remained unaffected when treated with different agents like fenofibrate, bezafibrate and metformin along with PA (Fig. 5a). PA treatment did not alter the Cpt2 expression, which is significantly increased when cells were co-treated with fibrates, however metformin did not show any effect (Fig. 5b).

PA-induced impairment of fatty acid oxidation is reversed by fibrates

Here the status of FAO upon the treatment of fibrates in the presence or absence of PA was measured according to the methods described earlier. The insulin-mediated FAO was reduced by PA-treatment and it was significantly recovered (~50% increased) upon treatment with fibrates (Fig. 6).

Fibrates restored PA-mediated downregulation of glucose uptake in myotubes

Various studies indicated that HFD induced insulin resistance is a result of reduced Glut-4 translocation to the plasma membrane. Further, recent reports suggest that synthetic agonists for LXR, restore HFD-induced decrease in Glut-4 translocation and improves IR
Insulin-mediated radiolabelled glucose uptake was measured in PA-treated L6 and C2C12 myotubes in presence or absence of fenofibrate, bezafibrate and metformin (Fig. 7a-b). PA treatment reduced insulin-mediated glucose uptake. Bezafibrate, a PPAR pan-agonist, fenofibrate, a PPARα-agonist and metformin significantly restored glucose uptake. Fibrates, on the other hand, caused significant recovery of PA-induced reduction in Glut-4 protein expression and translocation to the cell surface (Fig. 7c-d).

Discussion

Coexistence of FFA-induced insulin resistance (IR) and mitochondrial dysfunction has been widely reported. FFAs have been shown to induce IR in target tissues, presumably by altering the expression of certain genes or signaling pathways. Among all FFAs, palmitic acid (PA) causes IR (Fig. 1, a-b) in muscle cell lines. Obesity associated IR results primarily from impaired mitochondrial FAO [37]. We hypothesized that PA-induced mitochondrial dysfunction with reduced FAO in myotubes occurs due to downregulation of key genes regulating FAO. Differential expression of a number of genes associated with mitochondrial metabolism was observed, including those coding for mitochondrial carrier proteins i.e. CPT isoforms (Fig. 4), both in vitro upon PA treatment (Fig. 5a-b). While CPT1b present in the outer mitochondrial membrane is responsible for the production of fatty acyl carnitine at the
cytosolic side, CPT2 transfers the fatty acyl-CoA to the mitochondrial matrix for β-oxidation [38, 39]. Thus, CPT1b and CPT2 play crucial roles to transport FFA from cytoplasm to the mitochondrial matrix. PA-induced upregulation of Cpt1b results in excess palmitate accumulation in the interembrane space, as they cannot be transported across the inner mitochondrial membrane in the same rate by Cpt2, as the expression of the latter was not significantly changed. The accumulated palmitoyl CoA appears to flow back into the cytosol, increasing its cytosolic concentration (Fig. 2c). Excess cytosolic palmitoyl-CoA gets converted to ceramide (Fig. 3). Fibrates, especially fenofibrate facilitates the transfer of palmitoyl CoA to mitochondrial matrix by upregulating CPT-2 and restoring the ratio of CPT1b to CPT2 (Fig. 5). We have documented enhanced mitochondrial uptake of radiolabeled PA-derivatives and increased FAO rate following fenofibrate co-administration with radio labeled PA (Fig. 6).

Bezafibrate has been used successfully to improve FAO in CPT2 deficient patients [40]. In another report, a PPARδ agonist was shown to increase CPT2 mRNA level in the mitochondria, suggesting reversal of PA-induced impairment of FAO [41]. Fibrates bind to the cis-acting PPAR response elements, PPRE [42, 43], and activate CPT2 gene followed by normalization of transport of fatty acyl-CoA (Fig. 6). PA-induced increase in CPT1b and insignificant change of CPT2 expression prevents the entry of palmitoyl CoA into mitochondrial matrix. Excess PA accumulates in the cytosol and gets converted to ceramide, DAG and TAG; the latter promoting insulin resistance by activating TNFα and PKCθ-mediated serine phosphorylation of insulin receptors and serine307 phosphorylation of IRS-1 in skeletal muscle cells [44-47].

Here we show that fenofibrate, upon upregulating CPT2 expression, normalizes the PA-induced reduction in FAO rate and accompanying IR in myotubes. Myriocin, a ceramide synthesis inhibitor, partially restored FAO (data not shown) but did not reduce IR, possibly because it did not have any impact on CPT2 expression and FFA transport to mitochondria. This suggests that although accumulation of ceramide in myotubes may play a major role in IR, blocking its synthesis alone may not improve IR substantially. Etomoxir, a specific inhibitor of CPT1, reduced FAO by binding irreversibly to and inhibiting FFA transport to mitochondria [48-50] but did not improve PA-mediated IR, as it impaired fatty acid metabolism in cells. It also did not address the issue of cytosolic FFA accumulation and its subsequent conversion to ceramides (data not shown). We showed that fibrates and metformin could work in tandem to improve insulin-mediated glucose uptake even in presence of excess palmitate load (Fig. 7a). Fibrates exert their action by modulating the expression of CPT isoforms. As no CPT independent pathway is known to exist for FFA transport to the mitochondrial matrix, fibrate-induced changes in FAO is possibly mediated through its action on mitochondrial FA transport system.

Fibrate-induced restoration of CPT1b/CPT2 ratio reversed reduced FAO and IR by enhancing palmitoyl-CoA transport into the mitochondria thereby preventing its cytosolic accumulation and resultant decrease in ceramide synthesis. While PA-treatment of myotubes impaired insulin-mediated glucose uptake by increasing DAG/TAG/ceramide, addition of fibrates restored normal glucose uptake (Fig. 7a). We for the first time observed that fenofibrate can improve IR by improving FAO and reducing cytosolic FFA-accumulation. The remarkable improvement in mitochondrial FAO and insulin-mediated glucose uptake in myotubes by fenofibrate observed in our study is not replicated to a similar extent in clinical practice, possibly because of a much lower dose are used in human subjects due to safety concerns. A more potent and a safer PPARα agonist, like saroglitazar, may be the answer to palmitate induced lipotoxicity and insulin resistance in skeletal muscles in human subject with type2 diabetes in future [51, 52].

Our present data along with previous studies explain the molecular mechanisms underpinning PA or HFD-induced reduction in FAO and IR (Fig. 8). An optimal stoichiometric ratio of CPT1b/CPT2 appears to be an essential prerequisite for complete FAO in muscles. Palmitate, by upregulating CPT1b but not CPT2 alters this ratio, resulting in impaired FAO, cytosolic palmitoyl CoA accumulation, increased ceramide synthesis and impaired insulin-mediated glucose uptake. Fenofibrate abrogates palmitate-induced IR in myotubes by restoring CPT1b/CPT2 ratio, resulting in normalization of mitochondrial FAO and
prevention of cytosolic palmitoyl CoA accumulation and ceramide synthesis. Metformin increased glucose uptake by increase in Glut4 levels but did not address the reduced FAO or increased ceramide synthesis that continued to perpetuate IR. Fibrates, on the other hand, address the later issues adequately. We, therefore, suggest that obesity-linked IR may be better addressed by a combination of fibrates and metformin as this approach may not only improve insulin-mediated glucose uptake but also ensure optimal mitochondrial fatty acid uptake and oxidation, thereby preventing lipid induced insulin resistance.

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Disclosure Statement

The authors declare no conflicts of interest.

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