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Glucolipotoxic conditions induce β-cell iron import, cytosolic ROS formation and apoptosis

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Abstract

Type 2 diabetes (T2D) arises when the pancreatic beta-cell fails to compensate for increased insulin needs due to insulin resistance. Glucolipotoxicity (GLT) has been proposed to induce beta-cell dysfunction in T2D by formation of reactive oxygen species (ROS). Here, we examined if modeling glucolipotoxic conditions by high glucose-high free fatty acid (FFA) exposure (GLT) regulates beta-cell iron transport, by increasing the cytosolic labile iron pool (LIP). In isolated mouse islets, the GLT-induced increase in the LIP catalyzed cytosolic ROS formation and induced apoptosis. We show that GLT-induced ROS production is regulated by an increased LIP associated with elevated expression of genes regulating iron import. Using pharmacological and transgenic approaches, we show that iron reduction and decreased iron import protects from GLT-induced ROS production, prevents impairment of the mitochondrial membrane potential (MMP) and inhibits apoptosis. This study identifies a novel pathway underlying GLT-induced apoptosis involving increased iron import, generation of hydroxyl radicals from hydrogen peroxide through the Fenton reaction in the cytosolic compartment associated with dissipation of the MMP and beta-cell apoptosis.

Key Words

- intrinsic death pathway
- diabetes
- insulin secretion
- glucolipotoxicity

Introduction

Chronic nutritional overload is a very recent event in evolution, against which the development of cellular defense mechanisms has therefore not been advanced by selection pressure. In contrast, fluctuations in circulating substrate levels as a result of bouts of fasting and feeding have prevailed throughout human history and have selected for acute accommodations to nutritional fluxes, thereby defining the physiological regulation of metabolism. The pathophysiology of metabolic disorders is thus in part characterized by the adverse effects of inappropriate handling of nutritional overload, and in part by aberrant and chronic activation of normal metabolic pathways. Much is still to be learned about the molecular sensing, signaling and organelle relaying of substrate overload. Three main hypotheses have been proposed to explain the impairment of cellular replication, differentiation, function and survival caused by increased circulating levels of FFAs and glucose now by many referred to as glucolipotoxicity (GLT) (Muoio & Newgard 2008): oxidative stress mediated by excess generation of reactive oxygen intermediates (ROS); lipid partitioning, i.e. redirection of FFA metabolism from beta-oxidation
to build-up of toxic lipid metabolites and lipid storage and activation of low-grade inflammation. The molecular mechanisms regulating and executing the consequences of these major pathways are only partially understood.

In highly metabolically active cells such as cardiomyocytes and hepatocytes exhibiting high mitochondrial densities, the oxidative burden is typically counteracted by high mitochondrial oxidative capacity and by the expression of antioxidant enzymes (Lenzen et al. 1996, Lenzen 2017). In spite of lower mitochondrial density, the highly metabolically active pancreatic beta-cells are uniquely sensitive to oxidative stress due to inadequate antioxidant defense (Lenzen et al. 1996).

Iron-sulfur (Fe-S) proteins in the electron transport chain contribute to the generation of ROS from the mitochondria via the Fenton reaction (Thomas et al. 2009) and in the cytosol via NAPDH oxidase activity (Bedard & Krause 2007). Tissue iron overload confers risk for the development of diabetes (Ellervik et al. 2001, 2011, Jehn et al. 2004, Jiang et al. 2004, Montonen et al. 2012), and diabetes and the metabolic syndrome are associated with elevated circulating ferritin, a marker of cellular iron deposition (Ford & Cogswell 1999, Gabrielsen et al. 2012). Additionally, human and rodent beta-cells accumulate iron in conditions of increased circulating iron, as seen in hereditary hemochromatosis and transfusional iron overload, promoting islet apoptosis, decreased islet mass and impaired insulin secretion due to increased ROS production (Lu et al. 1991, Lu & Hayashi 1994, Cooksey et al. 2004). These clinical observations confirm that the beta-cell is particularly vulnerable to aberrant intracellular iron accumulation.

It is not known if GLT plays a causal role in enhancing beta-cell iron transport, thereby leading to excess ROS formation, beta-cell dysfunction and apoptosis. Therefore, we investigated this important science gap of considerable translational importance for the development of novel antidiabetic therapies targeting beta-cell iron handling.

**Materials and methods**

**Animal care**

Mice were housed with access to water and food ad libitum, in a 12-h light/darkness cycle (Hansen et al. 2012) (Supplementary materials and methods, see section on supplementary data given at the end of this article).

All the protocols were approved by the Animal Care Committee of the University of Toronto or Danish Animal Inspectorate.

**Islet isolation**

Islets were isolated after collagenase digestion and hand-picked (Supplementary materials and methods).

**Cell culture and treatment**

MIN6 cells were cultured in DMEM (Gibco) containing 4.5 g/L glucose, 10% fetal bovine serum (FBS, Gibco), 0.1% penicillin and streptomycin and 50µmol/L beta-mercaptoethanol (Gibco).

Isolated islets were cultured in RPMI 1640 supplemented with 10% FBS and 0.1% penicillin and streptomycin at 37°C with 5% CO₂.

For GLT treatment, culture medium was supplemented with or without endotoxin-free glucose and/or palmitate +/- oleate (both from Invitrogen) dissolved in 80% ethanol. Fatty acids were conjugated to BSA for at least 4 h at 37°C in a molar ratio of 3:1 in culture medium prior to use, and the final concentrations were palmitate (400 µM), combination of fatty acids palmitate (200 µM) and oleate (200 µM) and glucose 30 mM. Islets and MIN6 cells were treated with up to 72 h and human islets for 24 h. Culture medium supplemented with BSA and ethanol was used as control for GLT.

IL-1β (150 pg/mL) (BD Bioscience Pharmingen, San Diego, CA, USA) and IFN-γ (5 ng/mL) (R&D Systems) exposure was carried out for 24 h.

The iron chelator desferroxamine (DFO) (Sigma) was dissolved in water and deferasirox (D-sirox) (Santa Cruz Biotechnology) diluted in DMSO and added directly to the culture medium (Supplementary materials and methods).

**Glucose-stimulated insulin secretion perifusion and total islet insulin content**

**Glucose-stimulated insulin secretion**

Basal insulin secretion was established by pre-incubating islets or MIN6 cells for at least 1 h in 3 mM glucose Krebs–Ringer buffer (KRB). For stimulation, islets or MIN6 cell were incubated in KRB with the given concentrations of glucose for 30 min. KCl-induced insulin secretion was performed with 16.7 mM glucose and 30 mM KCl for 30 min. After glucose-stimulated insulin secretion (GSIS) experiments, islets were lysed in ethanol–HCl at −20°C for at least 24 h. Lysates were dried in a vacuum centrifuge, pellets were dissolved in ultra-pure water and DNA was measured by spectrophotometry using NanoDrop 2000 (Thermo Fisher).
Islet perifusion
Fifty islets from littermates were preincubated in KRB (3 mM Glc) for 2 h in KRB prior to perfusion, and then perfused at a flow rate of 1 mL per minute (Hansen et al. 2012). Total islet insulin was extracted from 10 to 20 islets lysed in ethanol–HCl at −20°C for at least 24 h. Insulin was measured using an insulin homogeneous time-resolved fluorescence kit according to manufacturer’s protocol (Cisbio, Codolet, France) or insulin ELISA (Kekow et al. 1988).

Calcein assay
Islets were incubated in imaging buffer with 0.25 µM calcein-AM (Molecular Probes) for 1 h. Fluorescence was detected and analyzed as described (Hansen et al. 2012) and Supplementary materials and methods. Total islet calcein loading was measured after chelation with 0.015 mM D-Sirox, and changes in LIP was calculated as difference between total calcein load and quenched calcein fluorescence.

Quantitative PCR
RNA was extracted using RNeasy Mini Kit (Qiagen), and RNA was measured using NanoDrop 2000 (Thermo Fisher). cDNA was synthesized using SuperScript II Reverse Transcriptase (Sigma), run with SybrGreen (Applied Biosystems) and analyzed on ViiA7 Real-Time PCR System (Applied Biosystems). Primers were purchased from TagCopenhagen (Copenhagen, DK) (Supplementary materials and methods).

Mitochondrial membrane potential and HIF1α translocation
Mitochondrial membrane potential (MMP) and HIF1α translocation were measured in whole islets as previously described (Chan et al. 2001, Cheng et al. 2010) (Supplementary materials and methods). In brief, islets were loaded with Rhodamine123 (Rhod123; Molecular Probes) (2.5 mg/mL) for 10 min in imaging buffer and the increase in MMP was measured as quenching of Rhod123 fluorescence. Fluorescence was recorded and analyzed as described in Supplementary materials and methods.

Apoptosis assay
Twenty-five islets per condition in duplicates were used for apoptosis assay. Apoptosis was detected using Roche Cell Death Detection ELISA (Roche) according to the manufacturer’s protocol.

Reactive oxygen species
Islets were loaded with CM-H2DCFDA (Molecular Probes) (10 µM) for 45 min in imaging buffer, washed once and ROS was detected on Flex Station 3 as described (Friberg et al. 2010) and Supplementary materials and methods.

Quantification of cytosolic and mitochondrial thiol oxidation
Islets were cultured, in batches of ten at different glucose concentrations and/or palmitate with or without DFO (0.1 mM), infected with adenovirus coding mito-roGFP1 or cyto-roGFP1 and transferred to a perfusion chamber in inverted microscope and fluorescence was measured every 30 s. After 20 min, 10 mM DTT was added to maximally reduce roGFP1, followed by 100 µM aldrithiol to maximally oxidize roGPF. The fluorescence ratio during the initial perfusion period was normalized to the ratio in DTT (set to 0%) and in aldrithiol (set to 100%). (Roma et al. 2012) and Supplementary materials and methods.

Statistics
Statistics were performed using Prism (GraphPad). Student’s t-test, and one- and two-way ANOVA with Bonferroni’s post hoc test were used to assess significance. P<0.05 was considered statistically significant.

Results
GLT increases the beta-cell labile iron pool
Islets isolated from mice fed a high fat diet (HFD) for 8 weeks had significantly increased LIP compared to islets from control-fed mice (Fig. 1A). GLT exposure in vitro for up to 72 h (Fig. 1B), but not palmitate alone (Supplementary Fig. 1A), significantly increased the islet LIP after 48 h compared to vehicle-treated islets. Of note, a combination of palmitate and oleate, considered to be a more relevant model of GLT in vivo, significantly increased the LIP in isolated human islets (Fig. 1C) to an even higher degree than the pro-inflammatory cytokines IL-1β and IFN-γ (Fig. 1D), previously shown to upregulate the iron transporter divalent metal transporter (DMT) 1 in human islets (Hansen et al. 2012).

To verify that the increase in islet LIP was beta-cell derived, we exposed insulin-producing mouse MIN6 cells to palmitate for up to 72 h. We did not manipulate the glucose concentration in MIN6 cell experiments, since MIN6 cells are normally grown in 25 mM glucose.
Palmitate significantly increased LIP in MIN6 cells after 24 h of exposure (Fig. 1E). This increase in beta-cell LIP was associated with decreased glucose-induced insulin secretion and increased ROS production and apoptosis (Supplementary Fig. 1B, C and D).

Next, we investigated if the GLT-mediated increase in the beta-cell LIP was associated with changes in expression of iron-handling genes in MIN6 cells exposed to palmitate for up to 24 h (Fig. 1F and G and Supplementary Fig. 1E, F and G). Palmitate significantly increased expression of the iron import genes DMT1 and transferrin receptor (Trfr) after 18 and 24 h, while the iron storage gene ferritin heavy chain (Fth) was transiently downregulated at 6 h. Ferritin light chain (Fli) and the iron export gene ferroportin (Fpn) were not regulated by palmitate exposure. Since increased ROS production has been suggested to induce beta-cell dysfunction through altered transcriptional activity (Prentice et al. 2014), we measured the expression of the beta-cell transcription factor pancreatic and duodenal homeobox 1 (Pdx1). Pdx1 was transiently downregulated after 6 h of palmitate exposure, while insulin 1 (Ins1) gene expression was unaffected (Supplementary Fig. 1H and I), suggesting that GLT-induced transient differences in transcriptional activity are not responsible for the observed differences in GSIS.

**Iron-mediated beta-cell apoptosis is associated with an increase in cytosolic thiol oxidation**

To investigate the causal role of GLT-induced increases in intracellular beta-cell LIP for apoptosis, we used the pharmacological iron chelator DFO to decrease the LIP in islets. DFO reduced the beta-cell LIP in a concentration-dependent manner after 48 h of treatment (Fig. 2A), without impacting absolute levels of GSIS, islet insulin content, accumulated insulin secretion or HIF1α nuclear translocation (Supplementary Fig. 2A, B and C and data not shown). The stimulatory index of the GSIS from the iron-chelated islets was decreased as expected (Supplementary Fig. 2A). The impact of the beta-cell LIP on GLT-induced apoptosis was then investigated in isolated islets exposed to vehicle or GLT for 48 h in the presence or absence of DFO. GLT significantly increased islet apoptosis two-fold, which was completely prevented by co-treatment with DFO (Fig. 2B).

We next asked if an increase in the LIP in islets exposed to a moderate glucose concentration (G11), G11...
iron chelation using DFO (Fig. 2C). Islet mitochondrial thiol oxidation was significantly decreased by DFO at basal, G30 and G30_PA, whereas a 48-h exposure to high glucose or palmitate did not change the mitochondrial thiol oxidation (Fig. 2D).

DFO treatment protected against FFA-mediated dysfunction estimated by GSIS and KCl-induced insulin secretion in islets exposed to a combination of palmitate and oleate for 24 h (Fig. 2E) shown above to increase human islet LIP (Fig. 1C).

**Iron is required for normal mitochondrial function**

Tamoxifen-inducible beta-cell specific DMT1 KO (Cre+Floxo) islets (Supplementary Fig. 3A) had significantly decreased LIP (Fig. 3A) resulting in decreased ROS formation compared to WT (Cre-Floxo) islets (Fig. 3B).

Since cytosolic ROS production promotes mitochondrial superoxide generation that may impair mitochondrial function (Coughlan et al., 2009), we next investigated the change in MMP of isolated Cre-Floxo+ and Cre-Floxo− islets upon glucose stimulation (Fig. 3C). At 3 mM glucose, Cre-Floxo+ islets exhibited a significant

supplemented with palmitate (G11_PA), high glucose (G30) or G30 supplemented with palmitate (G30_PA) caused an increase of ROS in the cytosol or in the mitochondria (Fig. 2C and D). We first verified that DFO did indeed reduce GLT-induced LIP in a concentration-dependent manner in MIN6 cells (Supplementary Fig. 2D). Palmitate exposure for 48 h increased thiol oxidation in the cytosol independently of iron, since DFO co-treatment did not reduce cytosolic thiol oxidation (Fig. 2C). This was consistent with the inability of palmitate alone to increase the beta-cell LIP (Supplementary Fig. 1A). In contrast, high glucose in combination with palmitate increased islet cytosolic thiol oxidation, which was decreased by
Iron uptake mediates β-cell glucolipotoxicity

**Figure 4**
Beta-cell-specific DMT1 KO reduces GLT-mediated apoptosis ROS production and mitochondrial damage. (A) Apoptosis measurement using DNA-histone complex detection by ELISA in isolated Cre+Flox+ and Cre−Flox− islets exposed to GLT (400 µM palmitate and 30 mM glucose) or vehicle (BSA and ethanol). Means ± s.e.m. of four independent experiments, **P<0.01, Student’s t-test.** (B) ROS production in isolated islets of Cre+Flox+ and Cre−Flox− islets exposed to GLT (400 µM palmitate and 30 mM glucose) or vehicle (BSA and ethanol) measured by H$_2$DCF fluorescence. Means ± s.e.m. of three independent experiments, **P<0.01 and **P<0.001 Vehicle Cre−Flox− vs GLT Cre−Flox−, **P<0.01 GLT Cre+Flox+ vs GLT Cre−Flox−, Student’s t-test.** (C) Insulin secretion measured by perfusion in isolated Cre−Flox− and Cre+Flox+ islets exposed to GLT (400 µM palmitate and 30 mM glucose) or vehicle (BSA and ethanol). Left panel, perfusion traces. Middle panel, stimulatory index of islet perfusion experiment. Right panel, total islet insulin after perfusion. Graphs show means ± s.e.m. of four independent experiments, **P<0.01, Student’s t-test.** (D) MMP measurements using Rhod123 in isolated Cre−Flox− islets exposed to GLT (400 µM palmitate and 30 mM glucose) or vehicle (BSA and ethanol). Left panel, means ± s.e.m. of fluorescence traces from islets analyzed, n=5–7. Right panel, means ± s.e.m. AUC of fluorescence traces normalized to time of n=4–5 independent experiments, *P<0.05, **P<0.01, Student’s t-test.** (E) MMP measurements using Rhod123 in isolated Cre+Flox+ islet exposed to GLT (400 µM palmitate and 30 mM glucose) or vehicle (BSA and ethanol). Left panel, means ± s.e.m. of fluorescence traces from islets analyzed, n=4–5. Right, means ± s.e.m. AUC of fluorescence traces normalized to time of n=4–5 independent experiments, *P<0.05, **P<0.01, Student’s t-test.

hyperpolarization of the MMP compared to Cre−Flox− islets, confirming that the decreased GSIS observed in Cre−Flox− islets, is due to inability to increase the MMP upon high glucose exposure. The MMP of Cre−Flox− and Cre−Flox− islets were similar when stimulated with 20 mM glucose, as well as when the mitochondria were completely depolarized using NaN$_3$.

**DMT1 KO protects from GLT-induced islet apoptosis associated with decreased ROS formation and preservation of the MMP**

GLT-induced islet apoptosis was completely inhibited in Cre−Flox− islets compared to Cre−Flox− islets (Fig. 4A) and correlated with decreased GLT-induced ROS formation (Fig. 4B). Additionally, since lipotoxic exposure of islets promotes attraction of pro-inflammatory macrophages to the islets to facilitate islet dysfunction (Eguchi et al. 2012), we investigated islet secretion of IL-6, TNF-α and MCP-1 (Supplementary Fig. 4A, B, C, D, E, and F). We did not observe upregulation of the cytokines or the chemoattractant by GLT; however, DFO treatment significantly reduced the production of MCP-1. Iron chelation with DFO, but not DMT1 KO, significantly increased islet production of TNF-α, suggesting that this action is not associated with iron-chelating properties of DFO.

Since Cre−Flox− islets were completely protected from apoptosis induced by 48 h of GLT exposure, we wanted to investigate if Cre−Flox− islets had preserved secretory function. The stimulatory index of the GLT-exposed DMT1 KO islets was significantly increased compared to GLT-exposed WT islets (Fig. 4C), showing that DMT1 KO in addition to protecting from GLT-induced apoptosis improves the ability of the islets to respond to glucose.
We next wanted to investigate if reduced ROS formation in Cre^{+}Flox^{−} islets protected mitochondrial function by measuring islet MMP after 48-hour exposure to GLT (Fig. 4D and E). GLT did not affect the MMP at 3 mM glucose in Cre^{−}Flox^{−} islets; notably however, at 20 mM glucose, Cre^{−}Flox^{−} islets exposed to GLT had significantly decreased MMP compared to vehicle-exposed Cre^{−}Flox^{−} islets as measured by the area under the curve. In Cre^{−}Flox^{−} islets, 48-hour exposure to GLT neither affected the MMP at 3 mM glucose nor the MMP at 20 mM glucose compared to vehicle-treated Cre^{−}Flox^{−} islet, suggesting that protection from cytosolic ROS formation prevented mitochondrial damage and subsequent impairment in MMP.

Discussion

In the present study, we investigated the mechanisms of GLT-induced beta-cell ROS production and cellular dysfunction. We found that GLT specifically alters the iron metabolism in beta-cells both in vivo and in vitro resulting in apoptosis. GLT induced Dmt1 and Trfr mRNA upregulation in beta-cells, and this was associated with increased iron uptake, increased intracellular LIP, cytosolic ROS production, mitochondrial dysfunction and beta-cell apoptosis. Importantly, we observed an increase in islet LIP from islets isolated from mice fed HFD for 8 weeks, where severe beta-cell damage is yet to occur but dysfunction is progressing. This suggests that increases in LIP and ROS formation might precede apoptosis in vivo.

Our work is in line with previously published findings by ourselves and others, where decreased dietary iron or decreased iron uptake protects beta-cells from HFD-induced dysfunction (Cooksey et al. 2010, Hansen et al. 2012). However, this observation was recently challenged in a study claiming that palmitate-mediated beta-cell apoptosis is mediated through a reduction in LIP and that DFO promotes beta-cell apoptosis (Jung et al. 2015). Because DFO is a potent iron chelator and iron is an absolute growth requirement for insulin-producing cells (Nielsen 1989), it is not surprising to us that DFO induces beta-cell apoptosis, which we have in fact demonstrated in MIN6 cells at concentrations of ≥0.1 mM (JB Hansen & T Mandrup-Poulsen, unpublished data). It is therefore important to carefully titrate iron chelators for such studies.

Dmt1 and Trfr are likely upregulated by JNK activation and ER stress, since (1) they contain predicted binding sites for the transcription factors AP1 and XBP1 respectively; (2) AP1 activity is increased by glucotoxicity in a beta-cell line and (3) Xbp1 mRNA is increased in rat islets by palmitate exposure and by palmitate and oleate exposure in human islets, respectively (Cunha et al. 2008, Maedler et al. 2008). These observations also provide a potential mechanism for the synergistic effect of glucose and FFAs in inducing the LIP in mouse beta-cells.

From studies in hepatocytes, another highly metabolically active cell, it has been proposed that GLT-induced ROS formation is caused by physical interaction between the mitochondria and ER with release of calcium from the ER to the mitochondria, resulting in decreased mitochondrial function, increased mitochondrial ROS formation and JNK activation (Arruda et al. 2014). Importantly, our data suggest that GLT induces cytosolic ROS production, rather than increasing mitochondrial ROS production. This, however, does not exclude GLT-induced beta-cell ER–mitochondrial interactions via calcium signaling and mitochondrial ROS production post or prior to iron accumulation, since DFO and DMT1 have limited or no calcium affinity (Munoz et al. 2011, Ehrnstorfer et al. 2014).

Recently, iron uptake in metabolically unstressed human beta-cells was proposed to be carried out primarily via non-transferrin-dependent iron import by the plasma membrane protein ZIP14 (solute carrier family 39 member 14, SLC39A14), independently of DMT1 and TRFR (Coffey & Knutson 2017), suggesting the existence of two distinct pathways for iron uptake. We hypothesize a physiological role for DMT1 to maintain insulin secretion by controlling the LIP and thereby the ROS levels required to fine-tune insulin secretion driven by the stimulus–secretion coupling. Of note, we found that the iron chelator DFO decreased the islet mitochondrial but not the cytosolic thiol oxidation in the absence of metabolic stress. This suggests that under normal conditions, the cytosolic LIP and ROS production is minimal and their contribution to GSIS likely to be limited. In addition, it confirms previous findings that mitochondrial function and ROS production primarily contribute to GSIS (Leloup et al. 2009) and shows the importance of iron in maintaining mitochondrial ROS production.

A caveat for the in vivo translation of the findings in this study is the diverse reports regarding the cellular effects and mechanisms of glucotoxicity, lipotoxicity and GLT and their dependence on experimental contexts (type of cell line, species, animal model for ex vivo studies, duration or concentrations of exposures). It should be noted, however, that we observed protection against HFD-induced glucose intolerance associated with increased circulating insulin levels in mice with inducible beta-cell-specific deletion of DMT-1 (Hansen et al. 2012), providing.

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in vivo evidence for the validity of the in vitro observations in the present study.

We conclude that iron transport via DMT-1 is a novel central mechanism relaying GLT with generation of hydroxyl radicals from hydrogen peroxide through the Fenton reaction and ROS formation in the cytosolic compartment associated with dissipation of the MMP and beta-cell apoptosis. Taken together with the accumulating epidemiological evidence suggesting that iron overload leads to diabetes (Ellervik et al. 2011, Bonfils et al. 2015) and that patients with diabetes have increased tissue iron accumulation (Rajpathak et al. 2009, Gabrielsen et al. 2012), our findings highlight control of iron levels as an attractive novel treatment option for the prevention of diabetes. Clinical trials are warranted to validate this translation of our preclinical findings.

Supplementary data
This is linked to the online version of the paper at https://doi.org/10.1530/JME-17-0262.

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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