Iron Depletion by Deferoxamine Up-Regulates Glucose Uptake and Insulin Signaling in Hepatoma Cells and in Rat Liver

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Iron depletion improves insulin resistance in patients with nonalcoholic fatty liver disease and diabetes and also stabilizes the hypoxia-inducible factor (HIF)-1, resulting in increased glucose uptake in vitro. This study investigated the effect of iron depletion by deferoxamine on insulin signaling and glucose uptake in HepG2 hepatocytes and in rat liver. In HepG2 cells, deferoxamine stabilized HIF-1α and induced the constitutive glucose transporter Glut1 and the insulin receptor. Up-regulation of insulin receptor by deferoxamine was mimicked by the intracellular iron chelator deferasirox and the hypoxia inducer CoCl2 and required the HIF-1 obligate partner ARNT/HIF-1β. Iron depletion increased insulin receptor activity, whereas iron supplementation had the opposite effect. Deferoxamine consistently increased the phosphorylation status of Akt/PKB and its targets FoxO1 and Gsk3β, which mediate the effect of insulin on gluconeogenesis and glycogen synthesis, and up-regulated genes involved in glucose uptake and utilization. Iron depletion of Sprague-Dawley rats increased HIF-1α expression, improved glucose clearance, and was associated with up-regulation of insulin receptor and Akt/PKB levels and of glucose transport in hepatic tissue. Conversely, gluconeogenic genes were not affected. In rats with fatty liver because of a high-calorie and high-fat diet, glucose clearance was increased by iron depletion and decreased by iron supplementation. Thus, iron depletion by deferoxamine up-regulates glucose uptake, and increases insulin receptor activity and signaling in hepatocytes in vitro and in vivo. (Am J Pathol 2008, 172:738–747; DOI: 10.2353/ajpath.2008.070097)
major site for insulin clearance via InsR-mediated uptake and degradation. Genetic deletion of InsR in mice leads to severe insulin resistance, hyperglycemia, and fatty liver, a picture similar to that of patients with severe NAFLD.

Decreased expression of the hypoxia-inducible factors (HIFs), involved in iron and oxygen sensing, has been implicated in the pathogenesis of diabetes through the down-regulation of the expression of InsR, of the dependent kinase Akt/PKB, and of molecules involved in glucose utilization in pancreatic β-cells. Indeed, HIF-1α levels are regulated by a family of prolyl-4-hydroxylases, which require iron and oxygen. In the presence of iron and/or oxygen, HIF-1α is hydroxylated and is bound by the Von Hippel-Lindau tumor suppressor protein and targeted for proteasomal degradation. The reduction of oxygen tension or iron deficiency results in HIF-1α stabilization; the stabilized HIF-1α dimerizes with the obligate partner HIF-1β/ARNT and regulates gene transcription by binding to hypoxia-responsive elements in the promoters of several genes orchestrating the response to hypoxic conditions. Besides regulating erythropoiesis and angiogenesis, HIF-1α has also been shown to increase glucose uptake by inducing Glut1 and glucose metabolism, thus favoring a shift to anaerobic metabolism. We thus hypothesized that HIF-1α could represent a link between glucose and iron metabolism.

The aim of this study was to analyze whether iron depletion influences glucose uptake and InsR activity in hepatocellular cell lines characterized by physiological regulation of insulin signaling, and in an in vivo model of iron depletion. We chose to induce iron depletion by deferoxamine (Dfo), a clinically used compound derived from iron depletion. We chose to induce iron depletion by

**Experimental Procedures**

**Cell Culture**

Human HepG2 cells were grown in RPMI 1640 medium. Hepa-1 C4 mouse hepatocytes, not expressing HIF-1β/ARNT, and their wild-type control Hepa-1 C1C7 mouse hepatocytes were grown in ARNT, and their wild-type control Hepa-1 C1C7 mouse hepatocytes, isolated from embryonic livers of transgenic mice lacking InsR (InsR−/−) and wild-type controls were maintained in α-minimal essential medium supplemented with 1 mmol/L L-GLN, 200 mmol/L dexamethasone, and 4% FCS. Cells were maintained in a humidified atmosphere with 5% CO₂ at 37°C. Cell viability was assessed in triplicate by Trypan blue exclusion. Unless otherwise specified, cells were treated with 100 µmol/L Dfo, 150 µmol/L cobalt chloride (CoCl₂), 150 µmol/L ferric ammonium citrate (FAC), 25 µmol/L deferasirox (Novartis, Basel, Switzerland), 33 mmol/L glucose, and 0.33 µmol/L insulin for 24 hours before experiments.

**Iron Responsive Protein (IRP) Activity Assay**

Total IRP activity, reflecting intracellular free iron availability (the higher IRP activity, the lower iron availability), was measured by RNA band shift assay as previously described.

**Oxidative Stress Evaluation**

Oxidative stress was measured by protein carboxylation, measured by Oxyblot (Chemicon, Temecula, CA), according to the manufacturer’s instructions.

**Western Blot Analysis**

Cells were lysed in RIPA buffer containing 1 mmol/L Na-orthovanadate, 200 mmol/L phenylmethyl sulfonyl fluoride, and 0.02 µg/µl aprotinin. Equal amounts of total cellular proteins (50 µg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred electrophoretically to polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). Membranes were incubated with anti-HIF-1α, InsR, Akt/PKB, FoxO1, and Gsk3 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), and phosphorylated Akt/PKB (p-Akt), phosphorylated FoxO1 (p-FoxO1), and phosphorylated Gsk3β (p-Gsk3β) antibodies (Cell Signaling Technology, Danvers, MA). For quantitative analysis, gels were scanned and analyzed by Image J software provided by the National Institutes of Health (Bethesda, MD), as previously described.

**Isolation of Cellular RNA**

RNA was isolated by a guanidinium isothiocyanate-phenol-chloroform procedure using Trizol (Invitrogen).

**Quantitative Real Time-Polymerase Chain Reaction (PCR)**

First-strand cDNA was synthesized with equal amounts (1 µg) of total RNA, by the SuperScript first strand synthesis system (Invitrogen). Quantitative reverse transcriptase (qRT)-PCR was performed with TaqMan universal master mix (1×), plus the assays specific for the genes of interest (MyScience; Applied Biosystem, Foster City, CA). All of the reactions were performed in triplicate in the ABI Prism 7700 sequence detector, in a 25-µl final volume.

**Insulin Iodination and InsR Activity Assay**

Bovine insulin was purchased from Sigma, St. Louis, MO. Insulin (1.5 µg) was iodinated before each experiment with 18.5 MBq [125I]iodine (Amersham, Stockholm, Sweden) and 1,3,4,6-tetrachloro-3a,6a-diphenylglycouril (1 mg/ml, lodogen; Sigma), obtaining a specific radioactivity of 80,000 to 130,000 cpm/µg of protein. Cells were incubated for 60 minutes at 37°C with [125I]-insulin in the presence of a 100-fold excess of unlabeled hormone.
Unbound $^{125}$I-insulin was removed from the culture dishes by rinsing the cells three times with phosphate-buffered saline and the bound radioactivity measured by gamma-counter. Results represent the means and standard deviations of at least two independent experiments performed in triplicate.

**Northern Blot Analysis**

Northern blot was performed by standard methods. Briefly, the gel was blotted to nylon membrane (Amer sham), and the film densitometrically scanned to quantify the relative proportion of InsR mRNA. β-Actin probes were used as reference standards. Images were analyzed as above.

**RT-PCR of InsR mRNA Isoforms**

We performed PCR amplification according to Savkur and colleagues: the experimental protocol allows semiquantitative evaluation of InsR isoforms A and B. β-Actin was used as reference standard; gels were densitometrically scanned and analyzed as above.

**In Vivo Cell Imaging**

FoxO1-GFP was constructed in pEGFPN1 (Clontech Laboratories, Palo Alto, CA). FoxO1 from FoxO1-pCMV was subcloned into this vector. Cells expressing FoxO1-GFP were transiently transfected by FuGene6 (Roche, Mannheim, Germany) using 2.5 µg/ml DNA. We plated and observed cells in LabTek chambers (Nalgene). We captured confocal microscope images on a LSM 510 META microscope (Zeiss, Jena, Germany) using 488-nm laser excitation for GFP and 568 nm for rhodamine. Images were captured with a 63 × 1.2 water objective and an open pinhole to collect fluorescence from the entire depth of the cell.

**Animal Model**

Male Sprague-Dawley rats weighing 180 to 200 g (Charles River, Calco, Italy) were maintained at the Preclinical Research Center of the Ospedale Policlinico, Milan, Italy, in compliance with the Principles of Laboratory Animal Care (National Institutes of Health publication no. 86-23, revised 1985). They were fed ad libitum and kept in a climate-controlled room with a 12-hour dark-light cycle. Rats were either iron depleted by daily injections of 200 mg/kg deferoxamine mesylate (Novartis) or submitted to injections of solvent (0.9% saline), for 2 weeks. The experimental protocol was approved by the local ethical committee. Basal weight and glucose levels (measured by glucose-oxidase assay) were in the reference range (3.1 mmol/L, Ins), were incubated with an excess of a $^{32}$P-labeled iron-responsive element probe; RNA-protein complexes were separated on nondenaturing 6% polyacrylamide gels and revealed by autoradiography. The corresponding percent cell viability, evaluated by Trypan blue, is indicated at the bottom. B: Protein carbonylation by Oxyblot in cells treated with FAC, Dfo, and untreated. DNP+, derivatization to 2,4-dinitrophenylhydrazone; DNP−, negative control.

**Results**

**Effect of Iron Manipulation on Intracellular Iron Pool, Cell Viability, and Oxidative Stress**

Dfo and FAC did not significantly affect cell viability at the concentrations used in this study (up to 120 µmol/L and 2500 µmol/L, respectively) (Figure 1A). Modulation of intracellular iron status by Dfo and FAC treatment was...
confirmed by measuring total IRP activity, which increases with decreasing intracellular free iron availability (Figure 1A). IRP activity increased in HepG2 cells treated with Dfo in a concentration-dependent manner, with a 62 ± 9% increase at 100 μmol/L ($P < 0.05$). Conversely, FAC addition decreased IRP activity, with a 43 ± 10% decrease at 150 μmol/L ($P < 0.05$). Glucose or insulin addition had no effect on IRP activity. Protein carbonylation levels were increased by FAC (150 μmol/L), but were not decreased by DFO (100 μmol/L) (Figure 1B).

**Iron Depletion Stabilizes HIF-1α and Increases Glut1 and InsR Expression**

Treatment of HepG2 cells for 24 hours with either Dfo or the hypoxia mimicker CoCl$_2$ (150 μmol/L) resulted in HIF-1α stabilization and increased InsR mRNA and protein levels. The effect of Dfo and CoCl$_2$ appeared to be additive (Figure 2A). We then analyzed the effect of Dfo on InsR in Hepa-1 hepatocytes lacking Hif-1/ARNT (C4), and in wild-type controls (C1C7). Dfo significantly increased InsR mRNA and protein levels in C1C7, but not in C4 Hepa-1 cells, suggesting that the presence of HIF-1β is required for the effect of Dfo on InsR expression (Figure 2B).

**Time Course Analysis of HIF-1α, Glut1, and InsR Expression after Dfo and CoCl$_2$ Treatment**

Next, we evaluated the time course of HIF-1α, Glut1, and InsR expression in cells cultured in the presence of Dfo (100 μmol/L), CoCl$_2$ (150 μmol/L), and Dfo plus CoCl$_2$ compared to control cells. We observed HIF-1α stabilization after 6 hours of treatment with Dfo and/or CoCl$_2$ (Figure 3A), which was paralleled by increased Glut1 mRNA levels (Figure 3B), about fourfold after 24 hours of treatment. Up-regulation of InsR mRNA and protein levels appeared to be delayed compared to Glut1, starting 9 to 24 hours after treatment with Dfo and/or CoCl$_2$ (Figure 3, A and B). Taken together, these data suggest that InsR induction may not be directly related to HIF-1α binding to InsR promoter.

**Effect of Deferasirox on HIF-1α and InsR Expression**

To confirm that the effect of Dfo on InsR expression was attributable to iron depletion, we compared HIF-1α and InsR protein levels in HepG2 cells treated with Dfo, deferasirox (ICL670, 25 μmol/L), or a combination thereof (Figure 4). Deferasirox and Dfo increased HIF-1α and InsR levels to a similar extent, whereas the effect of combination treatment was enhanced, suggesting that the effect of Dfo on InsR expression was dependent on iron chelation, and that it may be enhanced by chelators targeting a different iron pool.

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**Figure 2.** Effect of iron depletion and hypoxia on glucose uptake and InsR expression. A: Effect of incubation with CoCl$_2$ (150 μmol/L) and Dfo (100 μmol/L) for 24 hours in HepG2 on InsR and Glut1 mRNA, evaluated by qRT-PCR, and on HIF-1α and InsR protein levels. *$P < 0.05$ versus untreated cells. B: Iron depletion (Dfo, 100 μmol/L, for 24 hours) increased InsR mRNA and protein levels in C1C7, but not in C4 Hepa-1 cells, suggesting that the presence of HIF-1β is required for the effect of Dfo on InsR expression (Figure 2B).

**Figure 3.** Effect of Dfo and CoCl$_2$ on the time-course expression of InsR. A: Time-course expression of HIF-1α and InsR protein levels in HepG2 hepatocytes untreated, treated with Dfo (100 μmol/L), CoCl$_2$ (150 μmol/L), or with Dfo (100 μmol/L) and CoCl$_2$ (150 μmol/L). *$P < 0.05$ versus untreated cells. B: Time-course expression (normalized mRNA levels) of Glut1 (left panel) and InsR (right panel), as evaluated by qRT-PCR. White bars, untreated HepG2 cells; gray bars, cells treated with Dfo (100 μmol/L); dashed bars, cells treated with CoCl$_2$ (150 μmol/L); black bars, cells treated with Dfo (100 μmol/L) and CoCl$_2$ (150 μmol/L). *$P < 0.05$, **$P < 0.005$ for treated versus untreated cells.

**Figure 4.** Effect of Deferasirox on HIF-1α and InsR expression. A: Iron depletion (Dfo, 100 μmol/L, for 24 hours) increased InsR mRNA and protein levels in C1C7, but not in C4 Hepa-1 hepatocytes. Results are the mean and SD of two independent experiments. White bars, controls; black bars, Dfo-treated rats. *$P < 0.05$ for Dfo induced up-regulation of InsR versus wild-type C4 hepatocytes.
Iron Availability Regulates InsR Expression and Activity in Hepatocytes

We next evaluated the effect of iron status on InsR expression and activity in HepG2 cells. We first analyzed the effect of iron status on InsR mRNA levels. Northern blot analysis (Figure 5A) showed five major bands of InsR mRNA (9.2, 8, 7, 5.7, and 4.2 kb, corresponding to untranslated precursors and mature mRNAs). Densitometric analysis showed a twofold increase of InsR mRNA levels in cells treated with Dfo compared to untreated cells, and decreased expression in cells treated with FAC and glucose compared to untreated cells (P < 0.05). We next determined by RT-PCR whether iron manipulation could differentially affect the two alternatively spliced InsR isoforms, the A isoform, lacking exon 11, and B isoform containing exon 11, which encode for proteins characterized by different affinity, activity, and tissue distribution. Semiquantitative assessment indicated that Dfo treatment increased mRNA levels of both isoforms, but the fold induction appeared greater for the B isoform, whereas FAC specifically decreased the B isoform (Figure 5B).

We then evaluated InsR activity by a 125I-insulin binding assay. Addition of increasing amounts of 125I-insulin to untreated cells showed a saturable binding (bound radioactivity was evaluated by gamma-counter). Dfo increased by twofold InsR binding activity at the half-maximal concentration of 1.1 nmol/L, whereas iron addition as FAC or holo-transferrin reduced InsR activity by 30% (P < 0.005). The effect of Dfo was similar to that obtained by treating cells with 33 mmol/L glucose (44% reduction, P < 0.005), previously reported to down-regulate InsR.

Iron Depletion Affects Insulin Signaling Pathways and Glucose Utilization

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Iron Depletion Affects Insulin Signaling Pathways and Glucose Utilization

We then evaluated in HepG2 cells whether iron manipulation affects insulin-signaling cascade, focusing our attention on the Akt/PKB pathway. To simulate normal growth conditions, we first performed experiments in the presence of growth factors. In the presence of FCS, iron...
depletion obtained by Dfo treatment for 24 hours induced an increase in p-Akt/total Akt/PKB levels as compared to untreated cells (Figure 7A). We also observed a parallel Dfo-induced increase in the phosphorylation status of FoxO1 (Figure 7A) and of Gsk3β (Figure 6B). Akt/PKB targets that mediate the effect of insulin on gluconeogenesis and glycogen synthesis, respectively. To definitively exclude increased FoxO1 activity and gluconeogenesis, we looked at FoxO1-GFP intracellular localization in SV40 hepatocytes by in vivo cell imaging. Consistently with increased Akt/PKB activity, FoxO1-GFP was localized to the cytoplasm in Dfo-treated hepatocytes, as observed in the presence of growth factors (FCS), whereas it was nuclear in FCS-depleted cells (Figure 7C). In the absence of FCS, insulin treatment (0.33 μmol/L for 15 minutes) was more effective in inducing Akt/PKB activation in Dfo-treated HepG2 compared to control cells (Figure 7D). These data suggest that Dfo up-regulates Akt/PKB activity in HepG2 by enhancing insulin- and InsR-dependent Akt/PKB activity.

To look at the metabolic outcome, we analyzed the effect of iron status on the expression of genes involved in glucose uptake and glycolysis. Dfo treatment induced Glut1 and glucokinase (Gk), the latter involved in glucose activation for metabolic utilization in hepatocytes (Figure 8A, *P < 0.005), but not Glut2. Dfo also increased the expression of the glycolytic genes phosphofructokinase (Pfk) and glyceraldehyde-3-phosphate dehydrogenase (Gapd), whereas pyruvate kinase (Pk) mRNA levels were not affected (Figure 8B). Taken together, these results suggest that glucose uptake and utilization are increased in Dfo-treated hepatocytes.

Iron Depletion by Dfo Results in HIF-1α Stabilization and Increased Glucose Uptake, Hepatic InsR Expression, and Signaling in an in Vivo Model

At the end of Dfo or control treatment, nonfasting glucose levels (198 ± 29 mg/dl versus 185 ± 25 mg/dl) and body weight (260 ± 14 g versus 243 ± 13 g) were not signif-
significantly higher in the liver of Dfo-treated rats (Figure 9, C and D). These data indicate that iron depletion by Dfo was associated with increased glucose clearance, characterized by up-regulated InsR expression, insulin signaling, and glucose uptake by the liver.

**Effect of Iron Status in a Rat Model of Fatty Liver**

At the end of treatment HFD fed rats had significantly higher basal glucose levels compared to littermates (105 ± 18 versus 73.8 ± 38, *P < 0.05*). Basal glucose levels were not significantly different among controls, iron-depleted, and iron-supplemented rats. After intraperitoneal GTT, after 2 hours Dfo-treated rats had a lower increase, whereas iron supplemented rats had a higher increase, in glucose values compared to baseline levels versus controls (Figure 9E, *P < 0.05*).

**Discussion**

In this study, we investigated the effect of iron depletion by Dfo on glucose metabolism in hepatocytes using established cell lines characterized by physiological regulation of insulin signaling, and an in vivo model. Results indicate that treatment with Dfo induced cellular iron depletion comparable to that observed in humans, and increased glucose uptake, InsR expression, and insulin signaling. Our data suggest also that HIF-1α up-regulation plays a role in this process.

We hypothesized that iron depletion influences glucose metabolism by stabilizing HIF-1α, regulated at post-transcriptional level by iron status and oxygen tension. Both hypoxia and iron depletion by Dfo stabilized HIF-1α and stimulated InsR to a similar extent to Glut1, a known HIF-1α target. Up-regulation of InsR mRNA levels by Dfo resulted in increased binding and internalization activity, whereas iron addition, either transferrin-bound or nontransferrin-bound, reduced InsR activity to a similar extent to high-glucose concentrations, known to induce oxidative stress.
and InsR down-regulation.\textsuperscript{22,41} Semiquantitative assessment suggested that iron status influenced the levels of both InsR isoforms, but in particular of the B isoform, predominantly expressed in the liver and associated with increased signaling activity.\textsuperscript{36}

To determine whether the InsR increase enhanced insulin signaling, we next investigated the activation status of the Akt/PKB pathway, involved in the transduction of insulin signaling. Iron depletion induced an increase in p-Akt/Akt/PKB ratio, and of p-FoxO1 and p-Gsk3β levels, which mediate the effect of insulin on the inhibition of gluconeogenesis and the activation of glycogen synthesis, respectively. These data suggest that iron depletion may decrease hepatic glucose production and glycogen breakdown. Interestingly, it has been demonstrated that Akt/PKB activation is also involved in the insulin-mediated inhibition of apoptosis in hepatocytes through FoxO1 phosphorylation, suggesting that improved insulin sensitivity during iron depletion may decrease susceptibility to liver damage in patients with metabolic syndrome and NAFLD.\textsuperscript{42} Increased phosphorylation of Gsk3β induced by iron depletion may also contribute to stabilize HIF-1α in our model, as previously demonstrated in HepG2.\textsuperscript{43}

The expression of genes involved in glucose uptake and glycolysis was quantified to define the metabolic outcome. Dfo up-regulated Glut1, which mediates constitutive glucose uptake, and has recently been reported to be the most abundant glucose transporter in human tissues.\textsuperscript{27} In addition, Dfo induced Gk, the prevalent hexokinase in hepatocytes, and the rate limiting glycolytic genes Gapd and Pfk. Based on these data, it could be speculated that iron overload determines the suppression of the low basal HIF-1α activity in hepatocytes, determining InsR down-regulation and reduced insulin clearance and insulin signaling. The greater effect of Dfo compared to FAC on insulin signaling and gene expression is possibly related to the culturing conditions of cells: control cells cultured by standard methods were exposed to moderate iron concentration and high oxygen tension and, in fact, HIF-1α levels were lower than that observed in vivo in rats. Thus, although in vitro FAC increased oxidative stress, it could affect HIF-1α levels less than Dfo. These results may contribute to explaining the pathogenesis of the so-called hepatic iron overload-insulin resistance syndrome,\textsuperscript{44} a common cause of patients referral to specialized clinical centers.

To confirm these findings in vivo, we determined the effect of iron depletion on HIF-1α stabilization, InsR expression and signaling, and glucose uptake in Sprague-Dawley male rats. Dfo treatment resulted in iron depletion and increased hepatic expression of HIF-1α. We showed for the first time in vivo, in nonneoplastic cells,\textsuperscript{45} that iron depletion by Dfo determined a 10-fold increase in Glut1 mRNA levels. In addition, we observed increased InsR protein levels and Akt/PKB pathway activity, the latter partially explained by up-regulation of total Akt/PKB levels in Dfo-treated rats. These data are in agreement with recent findings in pancreatic β-cells, supporting a regulation mechanism of InsR, Akt/PKB, and glucose uptake by HIFs.\textsuperscript{27} Although mRNA levels of the rate-limiting gluconeogenic enzymes G6pc and Pck1 were not significantly affected, there was a trend for lower Pck1 expression in Dfo-treated rats. As a result, we observed a lower increase of glucose levels after intraperitoneal GTT in Dfo-treated rats in the presence of lower insulin levels, suggesting increased glucose clearance during iron depletion. Importantly, iron depletion ameliorated glucose clearance in a model of fatty liver characterized by increased basal glucose levels, whereas iron supplementation had the opposite effect, suggesting that iron status may affect the progression to hyperglycemia in the presence of hepatic insulin resistance.

In conclusion, the present findings indicate that iron depletion by Dfo affects glucose metabolism inducing glucose uptake and utilization and increasing InsR binding activity and signaling, and that the mechanism is associated with HIF-1α stabilization and requires the presence of HIF-1α/ARNT. A working model summarizing our results is shown in Figure 10. These results may contribute to explain the positive effect of iron depletion on insulin sensitivity in patients with NAFLD and diabetes,\textsuperscript{14,46,47} and are consistent with clinical findings indicating that the benefit is not restricted to patients with iron overload.\textsuperscript{16} Because HIF-1α activity can be pharmacologically modulated, additional studies are required to better characterize the role of this transcription factor in the regulation of glucose metabolism and in the pathogenesis of diabetes.\textsuperscript{27}

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