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Glucose acts as a regulator of serum iron by increasing serum hepcidin concentrations☆

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Abstract

Mutual clinical and molecular interactions between iron and glucose metabolism have been reported. We aimed to investigate a potential effect of glucose on iron homeostasis. We found that serum iron concentrations gradually decreased over 180 min after the administration of 75 g of glucose from 109.8±45.4 mg/L to 94.4±40.4 mg/L ($P<.001$; $N=40$) but remained unchanged in control subjects receiving tap water ($N=21$). Serum hepcidin, the key iron regulatory hormone which is mainly derived from hepatocytes but also expressed in pancreatic β-cells, increased within 120 min after glucose ingestion from 19.7±9.9 nmol/L to 31.4±21.0 nmol/L ($P<.001$). In cell culture, glucose induced the secretion of hepcidin and insulin into the supernatant of INS-1E cultures, but did not change the amount of hepcidin detectable in the hepatocyte cell culture HepG2. We additionally confirmed the expression of hepcidin in a human islet cell preparation. These results suggest that glucose acts as a regulator of serum iron concentrations, most likely by triggering the release of hepcidin from β-cells.

Keywords: Hepcidin; Glucose; β-cell; Serum iron

1. Introduction

The identification of the small 25-amino-acid peptide hepcidin as the central regulator of systemic iron homeostasis has shaped the understanding of mammalian iron metabolism over the past decade [1]. Hepcidin is derived mainly from hepatocytes and up-regulated in response to iron loading or inflammatory stimuli like interleukin 6 (IL-6) and down-regulated during iron deficiency, anemia or hypoxia [2]. Hepcidin serves as a negative regulator of serum iron concentrations by binding to the cellular iron exporter ferroportin (FPN1), inducing its phosphorylation, internalization and ubiquitin-mediated degradation. As a result, iron export from macrophages or hepatocytes is blocked [1]. In most forms of hereditary iron overload, the expression of hepcidin is inappropriately low. This leads to unregulated duodenal iron uptake and consecutive systemic iron accumulation with organ damage [3]. Elevated serum hepcidin concentrations are typical of inflammatory conditions and malignant diseases and play a central role in the pathogenesis of anemia of chronic disease [4].

Several clinical and molecular links between iron and glucose metabolism have been identified [5]. Typically, peripheral insulin resistance and impaired insulin secretion are found in patients with hemochromatosis and are associated with the degree of iron overload [6,7]. Moreover, an increasing incidence of type 2 diabetes has been linked to high body iron stores [8]. The frequently observed liver iron deposition with high ferritin and high-normal or mildly elevated transferrin saturation (TfS) in insulin resistant conditions is characterized by increased liver hepcidin mRNA expression and elevated serum hepcidin concentrations [9–12]. Impaired iron mobilization from liver cells and macrophages due to low expression of the iron exporter FPN1 seems to be an important mechanism underlying iron perturbations in insulin-resistant conditions, in particular nonalcoholic fatty liver disease [9,13].

Although the liver is the dominant source of circulating hepcidin, extrahepatic sites of hepcidin expression including the obese adipose tissue, alveolar and splenic macrophages, the heart and pancreatic β-cells have been identified [14–17]. The expression of hepcidin in the pancreas has been demonstrated in mice, rats and humans, but its

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potential physiological role in mammalian iron homeostasis is unknown. As insulin and hepcidin were previously localized to the β-cell granules [15], we hypothesized that glucose, the key stimulus for insulin secretion, might lead to a concomitant hepcidin release and thereby modulate serum iron concentrations. We therefore aimed to assess the effect of glucose ingestion on iron homeostasis *in vivo* and to evaluate the effect of glucose administration on hepcidin secretion from beta-cells *in vitro*.

2. Research design and methods

2.1. Study cohort

Subjects for oral glucose tolerance testing (oGTT; Salzburg Kolonkarzinom Präventionsinitiative) and controls were recruited from an ongoing screening colonoscopy program (the SAKKOPI Study) at a single center (Oberndorf Hospital) [18]. All study participants were subjected to routine medical examination, were considered healthy and did not have clinical or laboratory evidence of cardiac or renal insufficiency, cancer, systemic autoimmune disease, infectious or any known disorder of iron metabolism or diabetes. All study subjects had serum ferritin and TfS within the normal range, thus excluding subjects with iron deficiency, overload or underlying inflammation as modifiers of iron homeostasis. Written informed consent was obtained from all study participants to use data for scientific purposes, and the analyses were performed in accordance with the ethical standards set forth by the Helsinki Declaration of 1975 (revised in 1983). The study has been approved by the local ethics committee (Ethikkommission des Landes Salzburg).

2.2. Human islet cell preparation and liver biopsy samples

Islets were isolated from pancreas of a heart-beating, multiorgan donor using collagenase digestion and density gradient purification as reported previously [19]. Briefly, the cleaned pancreas was cut into two pieces and perfused with 0.5 mg Liberase (Roche Molecular Biochemicals, Indianapolis, IN, USA) under temperature- and pressure-controlled conditions and thereafter transferred to the digestion chamber. When approximately 50% free islets were detected, cells were collected in cooled centrifuge tubes filled with human albumin to inactivate the enzyme solution. Islets were purified in a Cobe cell processor (Cobe BCT, Lakewood, CO, USA) on a continuous Ficoll density gradient ranging from 1.06 to 1.17 for 5 min (Pan, Aldenbach, Germany). Islets were washed three times with cold M-199 medium and stored at −70°C. RNA was isolated, and reverse transcriptase polymerase chain reaction (RT-PCR) was performed as detailed below. Liver biopsy samples were used from a previous study from patients undergoing weight-reduction surgery [20]. We identified 18 subjects who were graded as level 0 fatty liver disease on ultrasound examination because hepcidin mRNA expression is well known to be different in fatty liver and normal liver tissue. Liver tissue samples were obtained from these subjects and stored in RNAlater (Ambion, Austin, TX, USA). Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany), and reverse transcription was performed as reported previously [21].

2.3. Laboratory evaluation

Following an overnight fast, a venous canula was placed in the antecubital vein at 8:00 a.m. Blood was drawn for a full blood count, determination of liver enzyme activities, serum iron status including ferritin, transferrin, transferrin saturation and serum iron, C-reactive protein, fasting glucose and lipids by standardized laboratory methods. Insulin was measured by Insulin Elecsys Analyzers (Roche, Vienna, Austria), and insulin resistance was calculated using the homeostasis model assessment (HOMA-IR; fasting insulin (μIU/ml)*fasting glucose (mmol/L)/22.5). C-peptide was determined using the ARCHITECT C-peptide-Assay (ABBOTT Diagnostics Division, Vienna, Austria). Standardized oGTTs were performed by administration of 75 g of glucose as a 500 ml solute. Control subjects received the same amount of tap water. Subsequently, blood was obtained at 30, 60, 120 and 180 min after glucose ingestion. Serum glucose, insulin and iron concentrations were measured immediately, and the remaining serum was stored at −70°C for the analyses of hepcidin and C-peptide.

2.4. Mass spectroscopy

Hepcidin-25 was quantified in human sera and in the supernatant of the human hepatoma cell line HepG2 according to a published protocol with slight modifications [22]. Briefly, serum samples (or supernatant) stored at −70°C were thawed to room temperature and vortex mixed, and after addition of 10 μl of 10% formic acid and 10 μl of internal standard (mouse hepcidin; 10 μg/ml; Bachem, Weil am Rhein, Germany) to 1-ml serum aliquots, samples were transferred to Oasis HLB 1-cc (30 mg) columns (Waters, Milford, MA, USA) preconditioned by the sequential addition of 1 ml methanol and water. Columns were washed with 1 ml water, 1 ml 30/75/5 (vol/vol/vol) methanol/water/ammonium hydroxide and then 1 ml water. Analytes were eluted with 1 ml of 90/10/0.1 (vol/vol/vol) methanol/water/formic acid into glass collection tubes and dried completely under nitrogen at room temperature. Samples were reconstituted with 100 μl of 0.1% formic acid in 10/90 (vol/vol) methanol/water and transferred to the G1367B (1200 Series) autosampler. Aliquots of 85 μl were injected onto a monolithic silica Chromolith RP-18e 100-3mm column (Merck, Germany). An Agilent 1200 Series HPLC system was used with 0.1% formic acid in 95/5 (vol/vol) methanol/water as mobile phase A and 0.1% formic acid in 5/95 (vol/vol) methanol/water as mobile phase B. The gradient applied at a flow rate of 400 μl/min was 15% B for 3 min, increasing to 90% B over 10 min, followed by a 5-min wash and reequilibration step. LC (liquid chromatography)/ESI (electrospray ionization)-MS/MS (tandem mass spectrometry) was performed using a QTRAP 3200 triple quadrupole mass spectrometer from AB SCIEX (USA), equipped with a TurbolonSpray ionization source, heated at 650°C and operated in positive ion mode at an ESI spray voltage of 5200 V. Settings used were curtain gas 20, ion source gas 1 (GS1) 80 and ion source gas 2 (GS2) 65. The ion transitions of MS/MS detection were m/z 698.1→354.1 for human hepcidin and m/z 918.6→331.4 for mouse hepcidin (internal standard). Analyst software version 1.4.2 was used for data collection and analyses. After confirming the absence of matrix interference with both human and mouse hepcidin, bovine serum (Gibco, USA) was used as a surrogate matrix to prepare calibrators and quality controls (QC). Seven calibrators at concentrations of 5, 12.5, 25, 50, 100, 250 and 500 ng/ml were run in duplicate at the beginning and end of each batch. For each analytical run, QC samples were analyzed at concentrations of 10, 50 and 150 ng/ml in duplicate. Overall recovery was determined by comparing the peak areas from injection of SPE extracts to blank SPE extracts spiked at the same concentration after the solid phase extraction (SPE) procedure in triplicate. Recovery rates at three tested concentrations were calculated to be 63.2%±10.4% for human hepcidin and 68.4%±8.7 % for the internal standard, respectively. Calibration curves were derived from ratios of the peak areas of hepcidin and the internal standard using $1/\chi^2$-weighted linear least-squares regression of the area ratio versus the concentration of the corresponding internal standard.

2.5. Cell culture experiments

INS-1E rat insulinoma or HepG2 human hepatoma cells were grown to confluence in 70-cm² culture flasks and seeded in six-well plates in RPMI 1640/8758 medium containing 11 mM glucose (Sigma Aldrich, St. Louis, MO, USA). In experiments addressing the role of glucose on hepcidin expression in INS-1E cells, RPMI 1640 without glucose (GIBCO Invitrogen, Carlsbad, CA, USA) was supplemented to the glucose concentrations indicated. Media were supplemented with 5% fetal calf serum, 100 mg/L gentamicin, 10 mM Hepes, pH 7.4, 1 mM sodium pyruvate and 50 μM 2-mercaptoethanol [23]. Insulin concentrations of the supernatant were measured using a Rat Insulin ELISA kit (Linco Research, St. Charles, MI, USA) [24]. Hepcidin secreted into the supernatant was determined by immunoblot analysis. Cells were washed twice with phosphate-buffered saline (PBS), incubated in RPMI 1640 containing 2 mM glucose for 30 min, washed again with PBS and incubated in RPMI 1640 medium containing 18 mM glucose for 180 min at 37°C. Control cells were kept in RPMI 1640 containing 2 mM glucose for the same period of time. After incubation, 500-μl aliquots of supernatants were vacuum-dried to approximately 20 μl, and Laemmli's loading buffer (10 μl) was added; samples were then subjected to sodium dodecyl sulfate electrophoresis in 16% (w/v) polyacrylamide gels followed by electrotransfer to an Amersham Hybond-P PVDF transfer membrane (GE Healthcare Ltd.). After blocking with 1× tris-buffered saline (TBS) containing 5% w/v nonfat dry milk for 1 h at 22°C, membranes were incubated with the primary antibody (Abcam AB30760, rabbit anti-hepcidin, activity to detect rat hepcidin according to the data sheet in Western blot analysis) overnight at room temperature. After washing, blocking and incubation with the secondary anti-rabbit horseradish peroxidase (HRP)-linked antibody (Cell Signaling Technology) for 1 h at room temperature, blots were exposed to SuperSignal West Dura substrate (Pierce Thermo Fisher Scientific), and chemiluminescence signals were recorded using the Kodak Imaging Station 2000 MM.

2.6. Fluorescence immunohistochemistry

Cells were seeded in six-well plates on collagen-coated coverslips and treated with PBS containing 0.5% bovine serum albumin (BSA), 0.2% Triton for 45 s, fixed with 1% paraformaldehyde for 15 min and washed 3× with PBS. Blocking was performed with PBS containing 0.5% BSA, 5% horse serum for 60 min. Samples were incubated with specific rabbit-anti-hepcidin antibody (Abcam, Cambridge, UK, AB30760; final concentration 1.5 μg/ml) and/or goat-anti-rat insulin antiserum (Abcam, Cambridge, UK, AB31906; diluted 1:200) overnight at 4°C, washed 3× with PBS and blocked prior to the addition of the respective fluorescent second antibodies (Alexa Fluor 568 goat-anti-rabbit for hepcidin, Alexa Fluor 488 goat-anti-guinea pig for insulin, final dilution 1:1000; Invitrogen, Carlsbad, CA, USA) for 60 min at room temperature. For nuclear staining, 2-4-diamidino-2-phenyindol dihydrochloride (DAPI; Molecular Probes, Eugene, OR, USA) was added at a final dilution of 1:500,000. Cells not incubated with the specific primary antibodies, but treated otherwise identically, were used to control for nonspecific staining by second antibodies. A Zeiss LSM T-PMT laser microscope (Carl Zeiss, Vienna, Austria) was used for confocal image analysis.

2.7. RT-PCR

Total RNA of INS-1E cells was extracted and reverse transcribed as described [24]. Transcripts of the genes encoding hepcidin, insulin-1 and insulin-2 were quantified by real-time PCR using the Sybr Green PCR master mix (Applied Biosystems, Vienna,

Austria). Primers were designed using Vector NTI software and obtained from ThermoScientific. Rat HAMP (hepcidin) sense: 5'-(55)TGTCTCCTGCTTCTCCTCCTG-3'; rat Hamp (hepcidin) antisense 5-(205)GAGGCATATGGGGAAGTTGGT-3'; rat insulin 1 sense: 5'-(226)AAGTGGAGGACCCGCAAGT-3'; rat insulin 1 antisense: 5'-(333) CACAATGCCACGCTTCTGCC-3'. Numbers in parentheses refer to primer positions relative to the translational start site of the respective cDNAs (GenBank). Results were normalized for mRNA expression of acidic ribosomal protein 0 (RPL0) mRNA (rat RPL0 sense fw: 5´-GGTACCATTGAAATCCTGAGCGAT-3´; rat RPL0 antisense: 5´-TTGCGGACACCCTCTAGGAAGC-3'; human RPL0 sense 5'-GGCACCATTGAAATCCTGAGT-GAT-3'; human RPL0 antisense 5'-TTGCGGACACCCTCCAGGAAGC -3' [24]. Human liver Hamp was determined using the Taqman assay Hs00221783_m1 and calculated relative to the expression of RPL0 as described [21].

2.8. Statistical analysis

SigmaStat 3.1 or SPSS (version 18.0) statistics packages were used. Data are presented as means±S.D. unless otherwise indicated. Differences between groups were calculated by Student's *t* test or Mann–Whitney *U* test in case of non-Gaussian distribution of parameters. Proportions were calculated using Fisher's Exact Test and χ^2 methods. Changes of glucose, C-peptide and hepcidin during the oGTT were calculated by a multivariate linear model adjusting for sex, age and body mass index (BMI). The Bonferroni correction was performed for multiple testing. Associations among parameters were calculated using nonparametric Spearman rank correlation analysis. The decline of serum iron concentrations during the observation period was assessed by paired *t* test.

3. Results

3.1. Glucose induces a rise of serum hepcidin and a decrease of serum iron concentrations in humans

In order to assess the effect of glucose on serum iron and hepcidin concentrations, we performed standardized oGTTs with 75 g glucose/500 ml water in 40 healthy subjects. Control subjects (N=21) drank the same amount of tap water without added glucose. General anthropomorphic and biochemical characteristics were similar in the experimental and the control group. These are summarized in Table 1. Of note, we did not find hepcidin concentrations to be different between our female and male study participants, which we attributed to the fact that there were mainly postmenopausal women in both the experimental and the control groups. Therefore, men and women were analyzed together.

Table 1
Clinical and biochemical characteristics of the study participants

Parameter	Subjects with OGTT	Control subjects
Number of patients	40	21
Female (%)	13 (32.8%)	9 (42.9%)
Age (years)	53.2±8.1	52.1±9.2
AST (10–32 U/ml)	24.0±12.2	25.2±11.9
ALT (10–32 U/ml)	26.8±17.9	26.1±18.1
AP (40–129 U/ml)	66.4±19.2	64.1±17.9
GGT (10–71 U/ml)	35.3±30.1	34.2±26.5
Uric acid (mg/dl)	5.2±1.4	5.1±1.4
Cholesterol (mg/dl)	213.9±35.8	210.7±35.2
LDL cholesterol (mg/dl)	135.6±39.7	137.2±36.1
HDL cholesterol (mg/dl)	65.2±19.4	62.3±20.2
Triglycerides (mg/dl)	114.4±45.8	121.0±51.4
Ferritin (ng/ml)	218.1±201.0	203.1±149.7
Serum iron (ng/ml)	112.3±50.1	106.9±55.7
TfS (%)	29.6±13.4	28.2±14.5
Fasting glucose (mg/dl)	97.2±10.2	96.2±10.8
HOMA-IR	2.05±1.71	1.99±1.79
BMI (kg/m²)	27.0±2.7	27.4±2.9
Hemoglobin (g/dl)	14.5±1.4	14.4±1.4
CRP (mg/dl)	0.29±0.37	0.21±0.30
Hemoglobin A1c (%)	5.78±0.25	5.81±0.22

The 40 subjects who underwent oral glucose challenge were similar to subjects who received an identical amount of drinking water with regard to the relevant clinical or biochemical characteristics. Data are presented as mean±S.D. unless otherwise indicated. Abbreviations: AST, aspartate aminotransferase; ALT, alanine aminotransferase; AP, alkaline phosphatase; GGT, gamma glutamyl-transpeptidase; HOMA-IR, homeostasis model assessment for insulin resistance; TfS, transferrin saturation; CRP, complement reactive protein.

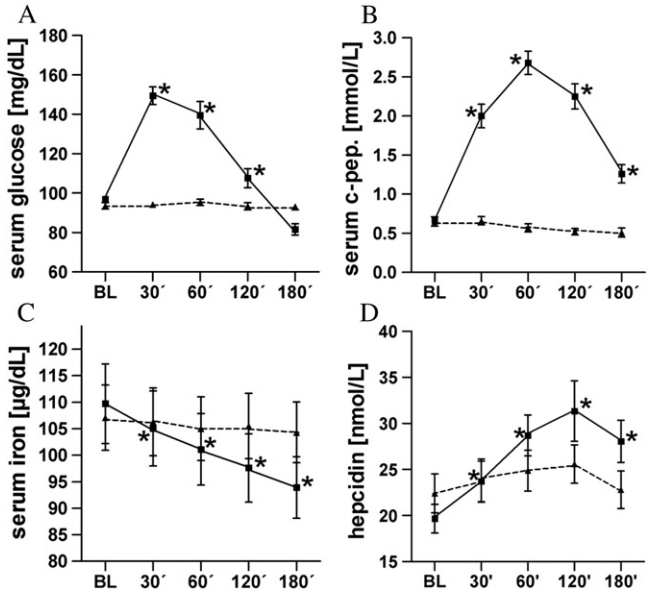

Fig. 1. Changes of serum glucose, C-peptide, iron and hepcidin concentrations in response to oral administration of 75 g glucose or water. Panels (A) and (B) show the expected increase in serum glucose and C-peptide concentrations demonstrating adequate β-cell response to ingested glucose (lines), whereas glucose and C-peptide remained unchanged in controls (dashed line). During the observational period, serum iron concentration decreased in the experimental group (C) along with an increase of serum hepcidin concentrations (D); no changes were observed in control subjects. Asterisks indicate significant difference of P<.01 compared to baseline. Data are depicted as means±S.E.M.

As expected, serum glucose, C-peptide and insulin concentrations increased and returned to baseline levels over 180 min in glucose-receiving subjects, while these parameters remained essentially unchanged in subjects ingesting 500 ml tap water (Fig. 1A and B, data for insulin not shown). Serum iron concentrations declined within 30 min after glucose ingestion and decreased further at all subsequent time points (109.8±45.4 mg/L to 94.4±40.4 mg/L; P<.001, baseline vs. 180 min; Fig. 1C), but remained stable at 107.2 ±23.3 mg/L vs. 105.1±25.1 mg/L (Fig. 1C) in the control group. Serum hepcidin concentrations increased in subjects receiving glucose, peaking at 120 min (19.7±9.9 nmol/L to 31.4±21.0 nmol/L, Fig. 1D). The increase in hepcidin concentrations in glucose-treated subjects compared to controls remained significant at 60 min (P=.025) and 120 min (P=.001) after the Bonferroni correction. In a multivariate linear model adjusting for sex, age and BMI, the increase in serum glucose, C-peptide and hepcidin concentrations over time in glucose-treated subjects was significantly different from that in control subjects (P<.001).

The decrease of serum iron concentrations over 180 min in glucose-treated subjects was correlated to the following parameters as calculated by Spearman rank correlation analysis: serum ferritin (R=0.323; P=.031); transferrin saturation at baseline (R=0.519; P<.001); AUC insulin (R=0.294 ; P=.050); AUC C-peptide (R=0.251; P=.091); hepcidin at 120 min (R=0.289; P=.060).

3.2. Hepcidin is expressed in rat insulinoma cells and released in response to glucose

We then aimed to determine whether the observed effects of glucose on serum iron and hepcidin concentrations could be due to the release of hepcidin from pancreatic β-cells using the rat insulinoma cell line INS-1E. We first confirmed the expression of

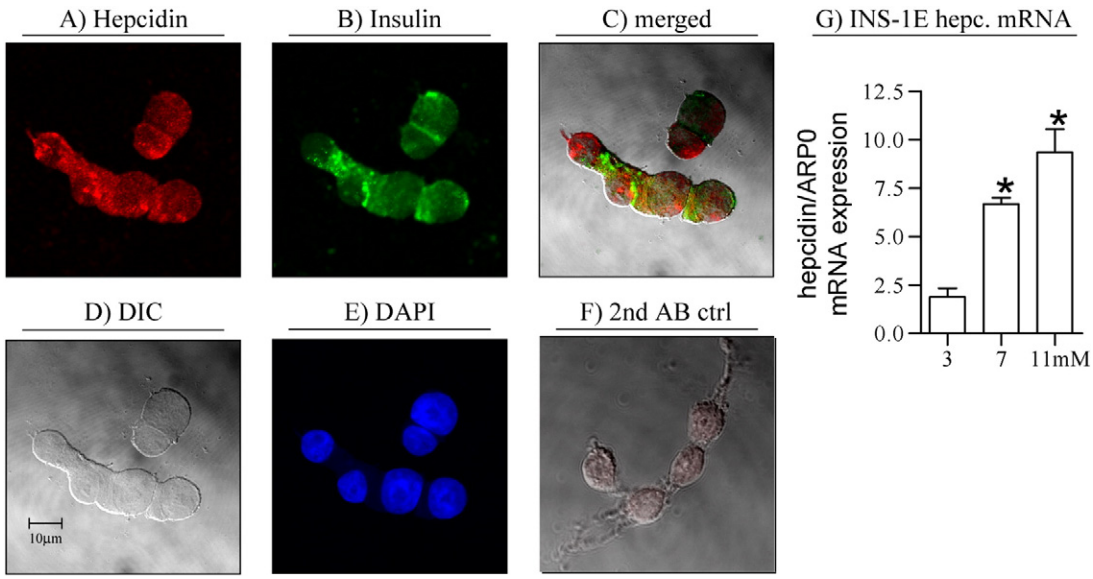

Fig. 2. INS-1E cells express hepcidin mRNA and protein. Confocal laser microscopy of INS-1E cells immunostained for hepcidin (red, A), insulin (green, B); merged images (C); differential interference contrast microscopy (D); nuclear staining with DAPI (E); nonspecific staining of cells in the absence of primary antibodies(F); mRNA concentrations of β-cell hepcidin cultured at different glucose concentrations (G). Results are presented as means±S.D. expression normalized to the housekeeping gene ARP0. Abbreviations: DAPI, 2-4-diamidino-2-phenyindol dihydrochloride; DIC, differential interference contrast; ARP0, acidic ribosomal protein 0.

hepcidin and insulin in INS-1E cells and detected both hepcidin mRNA and protein (Fig. 2A–F). The relative abundance of insulin mRNA was found to be approximately 10^4 higher than the mRNA expression of hepcidin in INS-1E cells. Hepcidin mRNA concentrations were similar at 7-mM and 11-mM glucose concentrations but were markedly decreased at 3-mM glucose culture conditions over 48 h (Fig. 2G).

Next, we assessed whether glucose, which is a potent stimulus for insulin secretion from β-cells, would also induce hepcidin release from INS-1E cells. The expected insulin secretion in response to glucose was confirmed by means of enzyme-linked immunosorbent assay (ELISA) (Fig. 3D). Fluorescence immunocytochemical analysis revealed lower intensity of hepcidin staining after glucose treatment for 180 min compared to cells kept on 3 mM glucose for the same period of time (Fig. 3A and B).Using immunoblot analysis, we observed an increase of a 10-kDa band in response to glucose in the supernatant after 120 min and 180 min (Fig. 3C). According to a previous report with a different hepcidin antibody, we assume that the 10-kDa band likely represents prohepcidin [25,26]. Moreover, a weak band of approximately 3 kDa corresponding to the molecular weight of hepcidin (2.8 kDa) was detected (Fig. 3C). We then aimed to determine whether this effect of glucose could also be observed in the hepatoma cell line HepG2. The same experimental protocol did not induce the release of hepcidin from HepG2 cells, i.e., hepcidin remained undetectable in the cell culture supernatant by mass spectrometry. And, likewise, immunocytochemical analysis did not reveal a difference in staining between high- and low-glucose treatments over 180 min (Fig. 3E and F).

In order to estimate the amount of hepcidin originating from β-cells under physiological circumstances, we determined the mRNA expression of hepcidin in a human pancreatic islet cell preparation and liver tissue relative to the housekeeping gene ARP0. The relative liver ($N=18$) compared to islet cell hepcidin mRNA abundance normalized to ARP0 was calculated to be 10.45±7.15 (ranging from 1.0 to 24.1), documenting a significant amount of hepcidin mRNA in the human islet cell preparation.

4. Discussion

In the current study, we found that, in healthy humans, glucose induced an increase of serum hepcidin concentrations independent of age, sex and BMI and, during the same period, serum iron concentrations gradually decreased. *In vitro* studies showed that glucose may achieve these effects by triggering the release of hepcidin from pancreatic β-cells.

Under physiological circumstances, serum iron concentrations are kept within narrow limits by elaborate mechanisms, and hepcidin serves as the main effector molecule [1]. These regulatory mechanisms must meet the requirements of iron-consuming tissues like the hematopoietic bone marrow and, at the same time, limit unbound and thus potentially toxic iron. Failure of these regulatory mechanisms is vividly illustrated by hereditary hemochromatosis where low hepcidin results in increased iron stores leading to progressive iron accumulation and damage to the liver, heart, endocrine organs and joints [27,28]. A rise of serum iron concentrations is counteracted by an up-regulation of hepcidin transcription and secretion from the liver via activation of the HJV/BMP/SMAD signaling pathway [29].

Our findings describe a hitherto unrecognized physiological mechanism in the regulation of serum iron concentrations which is triggered by the ingestion of glucose. The connection between glucose ingestion and the decline in serum iron concentrations reported here may be of particular relevance during nutrient-dependent fluctuations of serum iron concentrations. After intestinal absorption, iron is loaded to transferrin after FPN1-mediated export from the basolateral membrane of enterocytes [30]. With newly available iron entering the systemic circulation via the gut, it would be biologically plausible to buffer serum iron concentrations by simultaneously inhibiting iron export from liver cells and RES macrophages. Adaptation to postprandial nutrient excess represents a major physiological challenge, and multiple mechanisms facilitate absorption, systemic metabolism and storage of the main dietary contents, i.e., lipids, proteins or carbohydrates. Insulin secreted from the β-cell in response to glucose is the key anabolic hormone which regulates the incorporation of glucose, lipids and amino acids to their stores in liver, muscle or adipose tissue [31]. Administration of 75 g glucose represents a standardized and suitable test for the assessment of β-cell degranulation and the body's ability to handle glucose ingestion [32]. Interestingly, circadian variations of both serum iron and hepcidin concentrations have been reported previously [33,34]. One may speculate that the observations reported here may be involved in

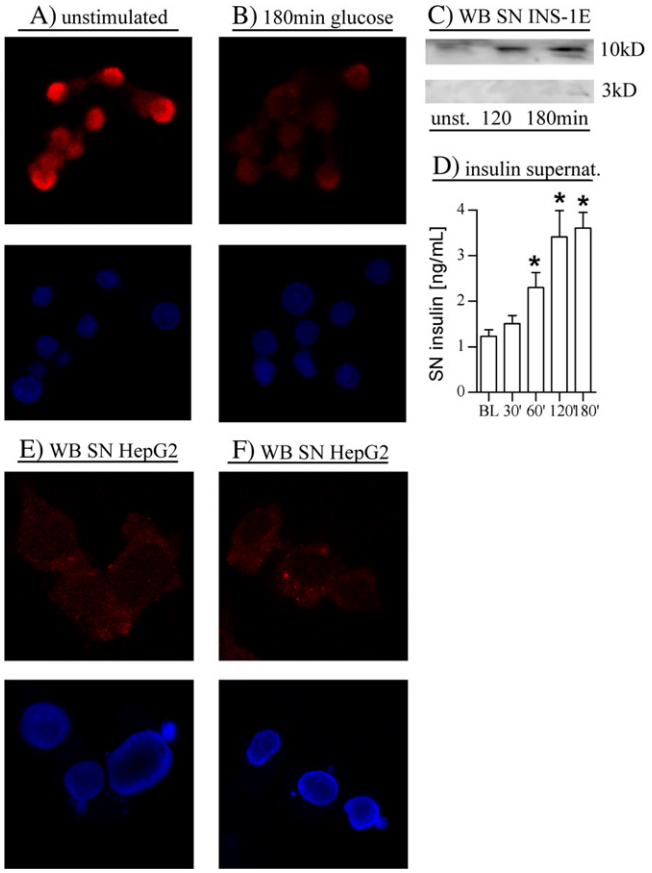

Fig. 3. Glucose induces the secretion of hepcidin from INS-1E cells but not HepG2 cells. Hepcidin staining in untreated INS-1E cells (2 mM for 180 min after culturing under standard conditions and 2 mM pretreatment for 30 min, A) and identically pretreated cells after 180 min at 18 mM glucose (B). Immunoblot analysis of hepcidin from the supernatant of INS-1E cells in response to glucose over 180 min with 10 kDa likely representing prohepcidin and a faint band at 3 kDa, which is the expected size of hepcidin (C). Insulin concentrations as determined by ELISA in the supernatant to assess adequate insulin release to glucose administration (D). Glucose did not change the amount of hepcidin detected by immunocytochemical analysis of HepG2 cells under the same conditions (E and F), and no hepcidin was detected by mass spectrometry. Abbreviations: WB, Western blot; supernat., culture supernatant.

these unexplained fluctuations of serum iron levels. We used standard oGTT over 3 h to assess the variations of serum iron concentrations in response to glucose and found a decline in serum iron which still was observed at the end of the investigation period. It would clearly be interesting to extend these studies over a longer time frame to assess the effect of glucose ingestion on the diurnal variations of serum iron concentrations.

It is a limitation to our findings that the pancreatic origin of the glucose-induced increase in serum hepcidin in the human situation is inferred from cell culture experiments. However, the synopsis of cell culture and *in vivo* results strongly supports a β-cell origin, but we currently cannot exclude that glucose administration may also trigger hepcidin release from other sources *in vivo*. However, *in vitro* glucose triggered the release of hepcidin from INS-1E cells, but did not change the amount of hepcidin released from the hepatocyte cell line HepG2. Nonetheless, identification of the definitive source of hepcidin after glucose ingestion will remain a major challenge in humans. We hypothesize that the particular compartmentalization of hepcidin to β-cell granules and its quick release in response to a nutrient ingestion could serve as a physiological mechanism to facilitate the postprandial adaption to iron absorption. Although the hepcidin mRNA concentration of a single human islet cell preparation was lower compared to the expression in liver tissue, the storage in β-cell granules may lead to a significant amount of hepcidin released over a brief period of time. In contrast, the quantitatively larger amount of hepcidin derived from the liver may operate as the long-term regulator of body iron stores. This is supported by the finding that, in cell culture, the amount of hepcidin released from HepG2 cells was not affected by glucose concentrations. Interestingly, by using immunoblot analysis, we detected mainly prohepcidin and less hepcidin in the supernatant of INS1-E cells. As the mechanisms of hepcidin processing in the β-cell have not been studied to date, it remains unclear whether INS1-E cells are not competent of prohepcidin cleavage, whether further degradation occurs in the circulation or if the observations made in INS1-E cells do not reflect the *in vivo* situation.

We also observed a strong correlation between serum ferritin concentrations and the decline of serum iron concentrations after glucose ingestion. Thus, either this mechanism may serve as a modulator of body iron stores or body iron stores may modulate the effect of glucose on serum iron concentrations. Along the same line of evidence, one may hypothesize that the observed down-regulation of hepcidin transcription in low-glucose β-cell culture could serve to augment iron availability during times of energy/nutrient restriction.

Over the past decades, insulin resistance and obesity, which are mainly known for their effects on lipid and glucose homeostasis, have become the most frequent causes of iron perturbations [35]. The relationship between iron homeostasis and obesity appears to be complex. First, insulin resistance is the most frequent cause underlying elevated ferritin concentrations, and removal of excess iron by phlebotomy has been reported to be beneficial [36]. Second, morbid obesity is often accompanied by iron deficiency and even anemia [37]. Both conditions, particularly in patients with nonalcoholic fatty liver disease, are characterized by increased serum hepcidin concentrations. The main source of elevated hepcidin concentrations in obesity and insulin resistance is the liver [9], but increased secretion from pancreatic islets may additionally contribute to the pathogenesis of insulin resistance associated iron perturbations. Elevated hepcidin concentrations may lead to both iron deposition in the liver and iron deficiency, via iron retention in the liver and decreased iron absorption from the gut. The number of patients studied here was not sufficient to calculate the difference between overweight and lean patients or between patients with and without fatty liver disease. However, preliminary results suggest that the decline of serum iron concentrations in response to glucose is indeed enhanced in subjects with NAFLD compared to subjects without fatty liver.

Although the results of this study suggest that the observed interaction between glucose and iron may serve primarily as a regulator of nutrient homeostasis, hepcidin is also a well-known regulator of iron metabolism under inflammatory conditions where hepcidin concentrations are generally increased and serum iron is low [38]. It will be an agenda to investigate how glucose affects hepcidin secretion under inflammatory conditions *in vivo* and *in vitro* and also to study the effect of proinflammatory cytokines like IL-1 and IL-6 on hepcidin expression in β-cells. Thus, the relevance of our findings to the pathogenesis of iron metabolism disorders such as the dysmetabolic iron overload syndrome, hemochromatosis, inflammation or iron deficiency will need further clarification.

In 2007, A. Pietrangelo proposed an intriguing concept of the regulation of systemic iron homeostasis as an endocrine feedback mechanism similar to the regulation of serum glucose concentrations by insulin [39]. As insulin is the key suppressor of serum glucose concentrations by interaction with its receptor on target tissues, hepcidin acts as the negative regulator of serum iron concentrations via interaction with FPN1 [15,39]. We here report an additional aspect of the insulin/glucose-like regulation of serum iron concentrations, namely, a quick lowering of the homeostatic component in the

bloodstream, i.e., iron, via secretion of the effector molecule, i.e., hepcidin, in response to glucose. Moreover, cell culture experiments suggest that the pancreatic β-cell may serve as the common source of both insulin and hepcidin after glucose absorption from the gut. These findings suggest that glucose, likely by triggering the release of hepcidin from pancreatic β-cells, may represent a novel regulator of serum iron concentrations.

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References

[1] Hentze MW, Muckenthaler MU, Galy B, Camaschella C. Two to tango: regulation of mammalian iron metabolism. Cell 2010;142:24–38.
[2] Zhang AS, Enns CA. Molecular mechanisms of normal iron homeostasis. Hematology Am Soc Hematol Educ Program 2009;2009:207–14.
[3] Weiss G. Genetic mechanisms and modifying factors in hereditary hemochromatosis. Nat Rev Gastroenterol Hepatol 2010;7:50–8.
[4] Weiss G. Iron metabolism in the anemia of chronic disease. Biochim Biophys Acta 2009;1790:682–93.
[5] Swaminathan S, Fonseca VA, Alam MG, Shah SV. The role of iron in diabetes and its complications. Diabetes Care 2007;30:1926–33.
[6] Hatunic M, Finucane FM, Brennan AM, Norris S, Pacini G, Nolan JJ. Effect of iron overload on glucose metabolism in patients with hereditary hemochromatosis. Metabolism 2010;59:380–4.
[7] McClain DA, Abraham D, Rogers J, Brady R, Gault P, Ajioka R, et al. High prevalence of abnormal glucose homeostasis secondary to decreased insulin secretion in individuals with hereditary haemochromatosis. Diabetologia 2006;49:1661–9.
[8] Jiang R, Manson JE, Meigs JB, Ma J, Rifai N, Hu FB. Body iron stores in relation to risk of type 2 diabetes in apparently healthy women. JAMA 2004;291:711–7.
[9] Aigner E, Theurl I, Theurl M, Lederer D, Haufe H, Dietze O, et al. Pathways underlying iron accumulation in human nonalcoholic fatty liver disease. Am J Clin Nutr 2008;87:1374–83.
[10] Barisani D, Pelucchi S, Mariani R, Galimberti S, Trombini P, Fumagalli D, et al. Hepcidin and iron-related gene expression in subjects with dysmetabolic hepatic iron overload. J Hepatol 2008;49:123–33.
[11] Ruivard M, Laine F, Ganz T, Olbina G, Westerman M, Nemeth E, et al. Iron absorption in dysmetabolic iron overload syndrome is decreased and correlates with increased plasma hepcidin. J Hepatol 2009;50:1219–25.
[12] Turlin B, Mendler MH, Moirand R, Guyader D, Guillygomarc'h A, Deugnier Y. Histologic features of the liver in insulin resistance-associated iron overload. A study of 139 patients. Am J Clin Pathol 2001;116:263–70.
[13] Aigner E, Theurl I, Haufe H, Seifert M, Hohla F, Scharinger L, et al. Copper availability contributes to iron perturbations in human nonalcoholic fatty liver disease. Gastroenterology 2008;135:680–8.
[14] Bekri S, Gual P, Anty R, Luciani N, Dahman M, Ramesh B, et al. Increased adipose tissue expression of hepcidin in severe obesity is independent from diabetes and NASH. Gastroenterology 2006;131:788–96.
[15] Kulaksiz H, Fein E, Redecker P, Stremmel W, Adler G, Cetin Y. Pancreatic beta-cells express hepcidin, an iron-uptake regulatory peptide. J Endocrinol 2008;197:241–9.
[16] Merle U, Fein E, Gehrke SG, Stremmel W, Kulaksiz H. The iron regulatory peptide hepcidin is expressed in the heart and regulated by hypoxia and inflammation. Endocrinology 2007;148:2663–8.
[17] Nguyen NB, Callaghan KD, Ghio AJ, Haile DJ, Yang F. Hepcidin expression and iron transport in alveolar macrophages. Am J Physiol Lung Cell Mol Physiol 2006;291:L417–25.
[18] Stadlmayr A, Aigner E, Steger B, Scharinger L, Lederer D, Mayr A, et al. Nonalcoholic fatty liver disease: an independent risk factor for colorectal neoplasia. J Intern Med 2011;270:41–9.
[19] Bertuzzi F, Grohovaz F, Maffi P, Caumo A, Aldrighetti L, Nano R, et al. Successful [correction of Succesful] transplantation of human islets in recipients bearing a kidney graft. Diabetologia 2002;45:77–84.
[20] Felder TK, Hahne P, Soyal SM, Miller K, Hoffinger H, Oberkofler H, et al. Hepatic adiponectin receptors (ADIPOR) 1 and 2 mRNA and their relation to insulin resistance in obese humans. Int J Obes (Lond) 2010;34:846–51.
[21] Hahne P, Krempler F, Schaap FG, Soyal SM, Hoffinger H, Miller K, et al. Determinants of plasma apolipoprotein A-V and APOA5 gene transcripts in humans. J Intern Med 2008;264:452–62.
[22] Li H, Rose MJ, Tran L, Zhang J, Miranda LP, James CA, et al. Development of a method for the sensitive and quantitative determination of hepcidin in human serum using LC-MS/MS. J Pharmacol Toxicol Methods 2009;59:171–80.
[23] Oberkofler H, Klein K, Felder TK, Krempler F, Patsch W. Role of peroxisome proliferator-activated receptor-gamma coactivator-1alpha in the transcriptional regulation of the human uncoupling protein 2 gene in INS-1E cells. Endocrinology 2006;147:966–76.
[24] Oberkofler H, Pfeifenberger A, Soyal S, Felder T, Hahne P, Miller K, et al. Aberrant hepatic TRIB3 gene expression in insulin-resistant obese humans. Diabetologia 2010;53:1971–5.
[25] Kulaksiz H, Gehrke SG, Janetzko A, Rost D, Bruckner T, Kallinowski B, et al. Pro-hepcidin: expression and cell specific localisation in the liver and its regulation in hereditary haemochromatosis, chronic renal insufficiency, and renal anaemia. Gut 2004;53:735–43.
[26] Kulaksiz H, Theilig F, Bachmann S, Gehrke SG, Rost D, Janetzko A, et al. The iron-regulatory peptide hormone hepcidin: expression and cellular localization in the mammalian kidney. J Endocrinol 2005;184:361–70.
[27] Allen KJ, Gurrin LC, Constantine CC, Osborne NJ, Delatycki MB, Nicoll AJ, et al. Iron-overload-related disease in HFE hereditary hemochromatosis. N Engl J Med 2008;358:221–30.
[28] Bridle KR, Frazer DM, Wilkins SJ, Dixon JL, Purdie DM, Crawford DH, et al. Disrupted hepcidin regulation in HFE-associated haemochromatosis and the liver as a regulator of body iron homoeostasis. Lancet 2003;361:669–73.
[29] Xia Y, Babitt JL, Sidis Y, Chung RT, Lin HY. Hemojuvelin regulates hepcidin expression via a selective subset of BMP ligands and receptors independently of neogenin. Blood 2008;111:5195–204.
[30] Anderson GJ, Frazer DM, McLaren GD. Iron absorption and metabolism. Curr Opin Gastroenterol 2009;25:129–35.
[31] Giugliano D, Ceriello A, Esposito K. Glucose metabolism and hyperglycemia. Am J Clin Nutr 2008;87:217S–22S.
[32] Mari A, Pacini G, Murphy E, Ludvik B, Nolan JJ. A model-based method for assessing insulin sensitivity from the oral glucose tolerance test. Diabetes Care 2001;24:539–48.
[33] Grebenchtchikov N, Geurts-Moespot AJ, Kroot JJ, den Heijer M, Tjalsma H, Swinkels DW, et al. High-sensitive radioimmunoassay for human serum hepcidin. Br J Haematol 2009;146:317–25.
[34] Third JL, Ryan MD, Sothern RB, Dawson S, McCormick JB, Hoffman HS, et al. Circadian distribution of iron and ferritin in serum of healthy and type 2 diabetic males. Clin Ter 2006;157:35–40.
[35] Bozzini C, Girelli D, Olivieri O, Martinelli N, Bassi A, De Matteis G, et al. Prevalence of body iron excess in the metabolic syndrome. Diabetes Care 2005;28:2061–3.
[36] Valenti L, Fracanzani AL, Fargion S. Effect of iron depletion in patients with nonalcoholic fatty liver disease without carbohydrate intolerance. Gastroenterology 2003;124:866–7.
[37] Cheng HL, Bryant C, Cook R, O'Connor H, Rooney K, Steinbeck K. The relationship between obesity and hypoferraemia in adults: a systematic review. Obes Rev 2012;13:150–61.
[38] Nairz M, Weiss G. Molecular and clinical aspects of iron homeostasis: from anemia to hemochromatosis. Wien Klin Wochenschr 2006;118:442–62.
[39] Pietrangelo A. Hemochromatosis: an endocrine liver disease. Hepatology 2007;46:1291–301.