A low-protein diet combined with low-dose endotoxin leads to changes in glucose homeostasis in weanling rats

Robert H. J. Bandsma,1,2,3 Cameron Ackerley,4 Khajag Koulajian,5,6 Ling Zhang,2 Tim van Zutphen,3 Theo H. van Dijk,3 Changting Xiao,5,6,7 Adria Giacca,5,6,7 and Gary F. Lewis5,6,7

1 Division of Gastroenterology, Hepatology, and Nutrition, The Hospital for Sick Children, Toronto, Ontario, Canada; 2 Physiology and Experimental Medicine Program, Research Institute, The Hospital for Sick Children, Toronto, Ontario, Canada; 3 Department of Pediatrics, Center for Liver, Digestive, and Metabolic Diseases, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands; 4 Department of Paediatric Laboratory Medicine, The Hospital for Sick Children, Toronto, Ontario, Canada; 5 Department of Physiology, University of Toronto, Toronto, Ontario, Canada; 6 Division of Endocrinology and Metabolism, Department of Medicine, University of Toronto, Toronto, Ontario, Canada; and 7 Banting and Best Diabetes Centre, University of Toronto, Toronto, Ontario, Canada

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Bandsma RH, Ackerley C, Koulajian K, Zhang L, van Zutphen T, van Dijk TH, Xiao C, Giacca A, Lewis GF. A low-protein diet combined with low-dose endotoxin leads to changes in glucose homeostasis in weanling rats. Am J Physiol Endocrinol Metab 309: E466–E473, 2015. First published July 7, 2015; doi:10.1152/ajpendo.00090.2015.—Severe malnutrition is a leading cause of global childhood mortality, and infection and hypoglycemia or hyperglycemia are commonly present. The etiology behind the changes in glucose homeostasis is poorly understood. Here, we generated an animal model of severe malnutrition with and without low-grade inflammation to investigate the effects on glucose homeostasis. Immediately after weaning, rats were fed diets containing 5 [low-protein diet (LP)] or 20% protein [control diet (CTRL)], with or without repeated low-dose intraperitoneal lipopolysaccharide (LPS; 2 mg/kg), to mimic inflammation resulting from infections. After 4 wk on the diets, hyperglycemic clamps or euglycemic hyperinsulinemic clamps were performed with [U-13C]glucose and [2-13C]glycerol to assess insulin secretion, action, and hepatic glucose metabolism. In separate studies, pancreatic islets were isolated for further analyses of insulin secretion and islet morphometry. Glucose clearance was reduced significantly by LP feeding alone (16%) and by LP feeding with LPS administration (43.8%) compared with control during the hyperglycemic clamps. This was associated with a strongly reduced insulin secretion in LP-fed rats in vivo as well as ex vivo in islets but significantly enhanced whole body insulin sensitivity. Gluconeogenesis rates were unaffected by LP feeding, but glycolysis was higher after LP feeding. A protein-deficient diet in young rats leads to a susceptibility to low-dose endotoxin-induced impairment in glucose clearance with a decrease in the islet insulin secretory pathway. A protein-deficient diet is associated with enhanced peripheral insulin sensitivity but impaired insulin-mediated suppression of hepatic glycolysis.

malnutrition; stable isotopes; insulin; glycemic clamps; oxidative stress

MATERIALS AND METHODS

Animals and diet. Pregnant Wistar rats were placed on regular chow. The offspring were kept with the mother until day 21, after which they were separated into two different diet groups: 1) a semisynthetic 5% protein [low-protein (LP)] diet and 2) a semisyn-
thetin control (CTRL; 20% protein) diet (Harlan, Madison, WI). The protein in the LP diet was replaced by starch, whereas all other components were kept constant. Since in preliminary studies LP-fed animals had reduced food intake (30% less than animals on the CTRL diet), the rats of the CTRL group were pair-fed the same amount as the rats on the LP diet. To exclude metabolic effects of a limited reduction in food intake, we initially added an additional control group of animals fed a regular chow diet ad libitum (CTRL ad libitum). The exact diet composition is provided in Table 1. Animals were kept on the diet for 4 wk. Two weeks into the diet, subgroups of rats on the CTRL and LP diets received an intraperitoneal injection of lipopolysaccharide (2 mg/kg, L2630; Sigma-Aldrich, St. Louis, MO) twice/wk (every 3–4 days). Rats were closely monitored for signs of illness, and weight was measured twice/wk.

**Surgeries.** Between days 21 and 23, a Micro-Renathane catheter was placed in the right jugular vein of the rats under general anesthesia. Rats were allowed 5–7 days to recover. Only rats that recovered uneventfully and lost <5% of their preoperative body weight were used for the clamp experiments.

**Hyperglycemic clamp experiments.** Rats were fasted overnight for 16 h, after which they received a bolus of a 37.5% glucose solution (750 mg/kg) through the right jugular vein. This was immediately followed by a continuous glucose infusion, which was started at 37.5 mg·kg⁻¹·min⁻¹ for 15 min and thereafter titrated to keep glucose concentrations at 17 mmol/l for a total duration of 90 min. Blood glucose measurements were performed by tail sampling every 5–10 min using a glucometer (One Touch Ultra 2; Lifescan, Milpitas, CA).

Additional small blood samples were obtained at t = 0, 2, 5, 10, 15, 30, 60, 80, and 90 min for measurement of insulin and C-peptide concentrations via enzyme-linked immunosorbent assays (Biovendor, Asheville, NC). After the clamp, animals were euthanized by a lethal dose of phenobarbital, after which blood and tissues were harvested. Six rats per group were used.

**Euglycemic hyperinsulinemic clamp experiments.** Animals were fasted overnight for 16 h, after which a baseline blood sample was taken from the tail. An infusion of [U-13C₆]glucose and [2-13C]glycerol was decreased to a rate of 200 g·kg⁻¹·min⁻¹ for 15 min and thereafter titrated to keep glucose concentrations at 17 mmol/l for a total duration of 90 min. Blood glucose measurements were performed by tail sampling every 5–10 min using a glucometer (One Touch Ultra 2; Lifescan, Milpitas, CA).

Additional small blood samples were obtained at t = 0, 2, 5, 10, 15, 30, 60, 80, and 90 min for measurement of insulin and C-peptide concentrations via enzyme-linked immunosorbent assays (Biovendor, Asheville, NC). After the clamp, animals were euthanized by a lethal dose of phenobarbital, after which blood and tissues were harvested. Six rats per group were used.

**Calculations.** Procedures for extraction of glucose from blood spots, derivatization of the extracted glucose, and gas chromatography-mass spectrometry (GC-MS) measurements of derivatives were all well established and performed as described previously (8, 45, 46). The measured fractional distribution measured by GC-MS (M6–M0) was corrected for natural abundance of 13C by multiple linear regressions, as described by Lee et al. (27) to obtain the mole fraction enrichment of mass isotopomers due to incorporation of infused labeled compounds, i.e., [2-13C]glycerol and [U-13C₆]glucose. Rate of glucose disposal, hepatic gluconeogenesis, and glycogenolysis were calculated as described before (46). The rate of endogenous production of plasma glucose [Ra(glc;endo)] was calculated as Ra(glc;endo) = [MPE(glc;M6)infusate/MPE(glc;M6)plasma – 1] × infusion(glc;M6), in which MPE(glc;M6) infusate is the mole percent enrichment of infused [U-13C₆]glucose, MPE(glc;M6) plasma is the mole percent enrichment of plasma [U-13C₆]glucose, and infusion(glc;M6) is the infusion rate of uniformly labeled [U-13C₆]glucose. Precursor enrichments (p[glc]) and fractional rate of gluconeogenesis ([fglc]) were calculated using mass isotopomer distribution analyses of glucose derivatives. The absolute gluconeogenic fluxes into plasma glucose [GNG(glc)] were calculated according to GNG(glc) = f(glc) × [total Ra(glc;endo) + infusion(glc;total)].

**Assessment of insulin secretion ex vivo.** Pancreatic islets were isolated from overnight-fasted rats using the Ficoll/Histopaque method, as described previously (33). The assessment of insulin secretion and islet insulin content has been described by Koulaaj et al. (23). Briefly, isolated islets of rats were preincubated for 1 h at 37°C in KRBH supplemented with 2.8 mmol/l glucose. Thereafter, five rat islets of approximately the same size were incubated in triplicate at 6.5 and 22 mmol/l glucose for 2 h at 37°C. Insulin concentration in the medium was analyzed with an ELISA kit (Merck, Darmstadt, Germany). Insulin concentration detection limits were between 0.5 and 100 ng/ml. The islets used for secretion were then subjected to acid ethanol extraction for measurement of insulin content. Islet reactive oxygen species (ROS) were measured using dihydrodichlorofluorescein diacetate (Life Technologies, Burlington, ON, Canada) followed by fluorescent microscopy (Olympus fluorescence BX51W1 microscope). The collected data were analyzed using the ImageMaster 3 software (43).

**Electron microscopy.** Pancreas was harvested immediately following euthanasia and fixed in 2.5% glutaraldehyde for electron microscopy (EM). For EM, tissues were postfixed in OsO₄ and embedded in Embed812 (SPI Supplies, West Chester, PA). Ultrathin sections were then prepared, mounted on grids, and stained with uranyl acetate and lead citrate. Images of β-cells from the islets were then captured with a charge-coupled device camera (AMT, Danvers, MA) and an electron microscope (JEM 1011; JEOL USA, Peabody, MA). At least 25 cells were examined from two animals from each group. The ratio of dark (more mature) to light granules was then determined from each cell for each group.

**Statistical analysis.** Values reported are means ± SD except for the EM morphometrics, islet ROS data, which are reported as means ± SE. Significance of metabolite and activity levels and fluxes was determined using two-way ANOVA with repeated measures where appropriate by SPSS Statistics package 21.0.0 (IBM SPSS, Armonk, NY). Differences were considered significant at P < 0.05.

## RESULTS

**Effect of LP diet and LPS on basic characteristics.** As expected, weaning animals fed an LP diet had drastically reduced body weight compared with CTRL diet pair-fed ani-

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Table 1. Compositions of the LP and CTRL diets

<table>
<thead>
<tr>
<th>Component</th>
<th>LP (5% Protein)</th>
<th>20% Protein</th>
<th>Regular chow</th>
</tr>
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<tbody>
<tr>
<td>Protein, g/kg</td>
<td>46</td>
<td>207</td>
<td>240</td>
</tr>
<tr>
<td>Methionine, g/kg</td>
<td>2.7</td>
<td>2.7</td>
<td>3.6</td>
</tr>
<tr>
<td>Starch, g/kg</td>
<td>392</td>
<td>251</td>
<td>383</td>
</tr>
<tr>
<td>Sucrose, g/kg</td>
<td>350</td>
<td>350</td>
<td>NA</td>
</tr>
<tr>
<td>Maltodextrin, g/kg</td>
<td>50</td>
<td>50</td>
<td>NA</td>
</tr>
<tr>
<td>Fat, g/kg</td>
<td>53</td>
<td>53</td>
<td>55</td>
</tr>
<tr>
<td>Energy, kcal/g</td>
<td>3.8</td>
<td>3.8</td>
<td>3.8</td>
</tr>
</tbody>
</table>

LP, low protein; CTRL, control; NA, not available. Composition for the regular chow could vary per batch since it is made from natural ingredients.
mals (average 67 vs. 201 g; see Table 2). Interestingly, protein restriction led to a decrease in food intake, i.e., the generation of a spontaneous hypocaloric intake that, to a lesser extent, also affected the pair-fed control animals in terms of body growth. The caloric intake was 40 ± 8, 39 ± 2, and 63 ± 12 kcal/day in the LP-fed, pair-fed CTRL, and ad libitum CTRL animals. The body weight of the CTRL and ad libitum group was 28% higher than the CTRL pair-fed diet group, and the difference between CTRL and other groups was even greater. Serum albumin concentration, a biochemical indicator of malnutrition, was severely reduced in LP-fed groups compared with CTRL pair-fed groups (average 26 vs. 39 g/l, P < 0.001). LPS administration was associated with reduced activity and food intake for ~48 h in both groups, although the effect appeared more pronounced in the LP-fed animals. There was no effect of LPS on serum albumin concentrations. Glucose and insulin concentrations after a 16-h fast were not significantly different (Table 2).

**Insulin secretion assessed in response to a hyperglycemic clamp.** The results of the hyperglycemic clamp are shown in Fig. 1A. The glucose concentration curves are shown in Fig. 1A. There was a lower glucose concentration after LP feeding compared with CTRL feeding (P < 0.01) during the first 15 min of the clamp, when all animals received a similar glucose infusion rate per body weight. Glucose concentrations at steady state, i.e., during the last 20 min of the clamp, were similar in all groups. Serum albumin concentration, a biochemical indicator of malnutrition, was severely reduced in LP-fed groups compared with CTRL pair-fed groups (average 26 vs. 39 g/l, P < 0.001). LPS administration was associated with reduced activity and food intake for ~48 h in both groups, although the effect appeared more pronounced in the LP-fed animals. There was no effect of LPS on serum albumin concentrations. Glucose and insulin concentrations after a 16-h fast were not significantly different (Table 2).

**Whole body insulin sensitivity, gluconeogenesis, and glycogenolysis.** To evaluate glucose metabolism and insulin sensitivity, we performed stable isotope experiments during the basal state and during a euglycemic hyperinsulinemic clamp. We evaluated the M6 enrichments to evaluate whether we had achieved an isotopic steady state. M6 enrichments were similar with 15.2 and 15.7% at 30 min before and at the end of the basal period, respectively. For the clamp period, enrichments were clearly at isotopic steady state with 18.9 and 19.0% at 30 min before and at the end of the clamp period, respectively. Glucose turnover was 16% in the LP group, consistent with reduced glucose clearance (Fig. 1B). The acute insulin secretory response during the first 15 min of the hyperglycemic clamp was blunted (P < 0.005) by LP feeding compared with CTRL feeding without an effect of LPS administration (Fig. 1C). Similarly, there was a lower steady-state insulin concentration in the LP-fed groups compared with CTRL-fed animals (P < 0.005; Fig. 1C). Similarly, there was a significantly lower steady-state insulin concentration in the LP-fed groups (2.3 ± 0.5 ng/ml) compared with CTRL pair-fed animals (3.9 ± 0.7 ng/ml) (P < 0.005) (Fig. 1C). There was no effect of LPS administration on insulin secretion. Results were similar, albeit less pronounced, for C-peptide concentrations (Fig. 1D). C-peptide concentrations in LP-fed animals were lower compared with CTRL-fed animals during the first 15 min of the clamp (P < 0.05). C-peptide concentrations during steady state were also lower in LP-fed animals (P < 0.05) without an effect of LPS. There was no effect of CTRL ad libitum feeding compared with CTRL pair-feeding for any of the parameters tested.

**Glucose-stimulated insulin secretion and islet insulin content in isolated islets.** Figure 2 shows the insulin response in isolated islets from rats in four different groups. Although the islet insulin content appeared to be lower after LP feeding, it did not reach statistical significance (P = 0.08; Fig. 2A). At 6.5 mmol/l glucose, there was an 84.1% reduction in insulin secretion after LP feeding, with no effect of LPS administration (P < 0.001; Fig. 2B). At 22 mmol/l glucose, insulin secretion was reduced by LP feeding by 50.5%, with a trend for even more reduced secretion after LPS administration (P < 0.005; Fig. 2B). Pancreatic β-cells have low antioxidant defenses and are susceptible to decreased function induced by ROS, and therefore, we determined the effect of LP feeding and LPS on ROS production. There was no significant effect of LP feeding or LPS on ROS concentrations (P = 0.1; Fig. 2C).

**Electron microscopy.** Light microscopy with insulin staining did not reveal major differences related to diet. In particular, there was no difference in the relative surface area of the islets to pancreas (data not shown). There was a lower dark/light granule ratio (index of granule maturity) in the LP-fed groups compared with the CTRL groups (Fig. 3). Administration of low-dose LPS did not have any effect on this ratio in either group of animals.

<table>
<thead>
<tr>
<th>Table 2. Basic characteristics of rats fed LP or CTRL diet with and without low-dose LPS</th>
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<tbody>
<tr>
<td><strong>CTRL Ad Libitum</strong></td>
</tr>
<tr>
<td>Body weight, g</td>
</tr>
<tr>
<td>Albumin, g/l</td>
</tr>
<tr>
<td>Glucose, mmol/l</td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
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Glucose and insulin levels were determined after 16-h fast; n = 6/group. *P < 0.001 vs. CTRL groups.
(138.7 ± 8.3 μmol·kg⁻¹·min⁻¹) (P = 0.07) and significantly higher during the steady-state clamp (333.1 ± 20.5 μmol·kg⁻¹·min⁻¹) compared with the CTRL groups (175.2 ± 14.1 μmol·kg⁻¹·min⁻¹) (P < 0.001; Fig. 4D). There was no effect of LPS administration on glycogenolysis rates.

DISCUSSION

Despite the consensus that severe malnutrition is still a widespread condition that is associated with a high mortality rate, few studies have focused on understanding the pathophysiological mechanisms that lead to disordered metabolic homeostasis in this condition. In the current project, we focused on studying hepatic and peripheral glucose metabolism and pancreatic islet function and morphology in a novel in vivo rat model of early postnatal severe malnutrition with or without chronic low-grade inflammation.

As expected, the low-protein dietary intervention led to a loss of growth, which has been reported previously (16, 42). This is consistent with what is seen in children with severe malnutrition, which is defined by a weight-for-height z-score of less than −3 (49). The main results indicate that 1) there is decreased responsiveness of pancreatic β-cells that is related to decreased basal and glucose-stimulated insulin secretion, with relatively preserved islet insulin content after LP feeding; and 2) severe malnutrition is associated with enhanced peripheral insulin sensitivity, appropriately suppressed hepatic gluconeogenesis but increased hepatic glycogenolysis, as evidenced during the euglycemic hyperinsulinemic clamp.

There have been a number of clinical reports indicating changes in pancreatic β-cell function in malnourished patients (14, 21, 39). Pancreatic function in these children was generally assessed using oral glucose tolerance tests. However, we recently reported severely impaired glucose absorption in malnourished children (9). Decreased absorption of glucose would ultimately also influence insulin secretion, making the use of an oral glucose tolerance test less reliable in malnourished children. There are also animal studies focusing on pancreatic β-cell function during malnutrition. Although some data have indicated a normal insulin response after a low-protein diet (16), most in vivo studies using oral or intravenous glucose tolerance tests (32, 35, 42) and isolated pancreatic islet (17, 38) data have shown decreased pancreatic β-cell response. In this study, we provide further in vivo and in vitro evidence for a decrease in pancreatic β-cell insulin secretion by using the gold standard for assessing pancreatic function, i.e., the hyperglycemic clamp technique.

Our data indicate that the reduced insulin secretory response is not related to a significant lack of insulin, as islet density and insulin content were not significantly reduced in the LP-fed animals. Our electron microscopy did reveal an increased presence of pale islet granules, probably representing an immature population of progranules (34). This is consistent with
data obtained from postmortem-acquired pancreatic tissue of severely malnourished children (12). Only a handful of studies have been published that examined the pathophysiological mechanisms responsible for pancreatic β-cell failure in malnutrition. Reduced insulin secretion in protein-malnourished mice might be related to alterations in the stimulus-secretion coupling of pancreatic β-cells, including more hyperpolarized membrane potential attributed to higher activity of ATP-sensitive potassium ion channels, thereby leading to reduced Ca\(^{2+}\) influx and an inhibition of insulin release (38). Limited animal data also suggested changes in the cAMP/protein kinase A regulation of the insulin secretion pathway (17) and possibly changes in insulin receptor and insulin receptor substrate activity (35).

In this study, we did observe an additional effect of LPS in the LP-fed group on glucose clearance during the hyperglycemic clamp. However, we could not detect a statistically significant effect of LPS on insulin secretion. LPS has been shown to rapidly decrease insulin secretion in isolated islets ex vivo (48). More recent data generated evidence that the effect of LPS is mediated through Toll-like receptor 4 (4). In addition, we and others have shown that impaired insulin secretion can be mediated through oxidative stress (24, 25, 33, 50). Pancreatic β-cells are relatively vulnerable to oxidative stress due to their relatively low free radical detoxifying and redox-regulating enzymes (31). Impairment of pancreatic β-cell function is ameliorated or prevented by antioxidant therapy in animal models (33) and in humans (51). In children, severe malnutrition is associated with oxidative stress (18, 30). However, although there was a trend for increased islet ROS levels especially in the LP group with LPS, we could not find a significant effect of either LP feeding or LPS. Although the
dose of LPS used in this study did affect glucose clearance, it is likely that the dose was not sufficiently high to strongly affect insulin secretion. The dose we used for this study was aimed at trying to mimic a low-grade chronic inflammation that is often clinically observed in malnourished children, e.g., related to tuberculosis, chronic intestinal infection. We intentionally did not want to create an overwhelming condition of sepsis that would also potentially mask any metabolic effects caused by malnutrition. It would have been interesting to evaluate different doses of LPS, but unfortunately, this was not feasible due to the labor-intensive property of the clamp procedures.

Whole body insulin sensitivity was found to be higher in LP-fed rats compared with regularly fed rats. Our study is the first to study insulin sensitivity using the gold standard, i.e., the euglycemic hyperinsulinemic clamp technique in malnutrition. Reis et al. (35) found normal glucose response during a glucose tolerance test despite decreased insulin response in malnourished rats, suggesting enhanced insulin action. Other animal and human studies using glucose tolerance tests also found some evidence for enhanced insulin sensitivity in malnutrition (3, 16, 39). The increased insulin sensitivity may be due to lower body weight and fat mass and/or may be adaptive to low insulin secretion. Intriguingly, repeated low-dose LPS administration had no effect on insulin sensitivity in our experiments. Sepsis and administration of LPS have been shown to induce insulin resistance both in animal studies as well as in human subjects (1, 47).

In this study, we did find evidence for some degree of hepatic insulin resistance. Although gluconeogenesis was similarly suppressed during the euglycemic clamp in both control and LP-fed rats, we observed increased rates of glycogenolysis during the euglycemic clamp in the LP-fed rats. As far as we know, no studies that have specifically addressed the regulation of hepatic glycogenolysis in childhood malnutrition exist. In conditions of insulin resistance, glycogenolysis and gluconeogenesis are usually suppressed to a similar extent (10). Although we are unable to confirm this, we cannot exclude that differences in corticosterone or catecholamines levels contributed to the different glycogenolysis rates between the groups.

There are several limitations to this study. First, our sample size was relatively small. It could be that the study was underpowered to detect between-group changes in islet insulin concentration and basal glycogenolysis. However, these values were physiologically close to each other anyway, so they likely would not have affected our main conclusions. In addition, although dietary calorie intake was similar among the animals, weight changes among the groups increased over time, leading to a higher caloric intake in the LP-fed animals when expressed per kilogram body weight. All kinetic analyses were expressed per kilogram body weight. If expressed per lean body weight, it could have influenced values for glucose infusion rates. However, we felt that, at least when related to hepatic glucose metabolism, expression per total body weight was acceptable, as liver weight per body weight was similar in all groups. Unfortunately, we did not have the possibility to determine...
body composition in our animals. Furthermore, the insulin dose used in the euglycemic hyperinsulinemic clamp study was relatively modest. A higher or loading dose of insulin would have likely led to a suppression of hepatic glycogenolysis in the control group but could have prevented us from finding a difference in glycogenolysis between the low-protein and control groups. The absence of an insulin-loading dose could very well have prevented us from reaching a complete metabolic steady state, which would affect glucose clearance rates and possibly serum FFA concentrations. However, we are confident that this would not have affected our conclusion on the difference found between the groups. We tried extensively to insert cannulas into carotid arteries, which would allow serial sampling of larger volumes for analysis of insulin concentrations to better determine whether a steady state was reached. We were able to insert them successfully, but we found that for the small malnourished animals we had to use such small diameter cannulas that it was not feasible to withdraw blood from them without clotting the lines. Tail sampling is more stressful for rats, especially if one needs to collect larger volumes of blood and manipulate the tail longer. For ethical reasons and because of the effect of stress on glucose homeostasis, we decided to take only small bloodspot samples for glucose and enrichment measurements and have one final blood draw at the time of euthanasia, which showed similar insulin concentrations in control and LP-fed animals.

In conclusion, our study shows that reduced insulin secretory response during severe malnutrition is compensated at least in part by increased peripheral insulin sensitivity. In addition, low-protein diet-induced malnutrition leads to an enhanced rate of glycogenolysis. The results of this study might explain the pathophysiology of hyperglycemia in children with severe malnutrition. Improving glucose tolerance in sick, severely malnourished patients could potentially lead to a decrease in mortality. However, in low-resource settings, interventions with insulin might not be feasible and potentially high risk due to the increased insulin sensitivity of malnutrition. Since impaired β-cell function in the context of refeeding could lead to hyperglycemia, whereas sudden improvements in insulin release with enhanced insulin sensitivity during refeeding may cause hypoglycemia, further studies are also required in a refeeding model. These results provide insight into the metabolic disturbances occurring during early postnatal malnutrition in vivo and could have important consequences for treatment of severely malnourished children.

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GRANTS

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DISCLOSURES

All authors report that there is no duality of interest related their contributions to this article.

AUTHOR CONTRIBUTIONS


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