Multigenerational Undernutrition Increases Susceptibility to Obesity and Diabetes that Is Not Reversed after Dietary Recuperation

Highlights
- Undernourished rats are protein / calorie-restricted for 50 generations
- Recuperation rats are generated by feeding normal chow for two more generations
- Undernourished and Recuperation rats show multiple markers of metabolic disease
- Metabolic / epigenetic alterations are not reversed following nutrient recuperation

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In Brief
In a rat model of undernutrition over 50 generations, closely mimicking human populations in developing countries, Hardikar et al. show that undernourished rats display metabolic abnormalities associated with epigenetic changes, which are not reversed following unrestricted access to normal chow in two subsequent generations.
Multigenerational Undernutrition Increases Susceptibility to Obesity and Diabetes that Is Not Reversed after Dietary Recuperation

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SUMMARY

People in developing countries have faced multigenerational undernutrition and are currently undergoing major lifestyle changes, contributing to an epidemic of metabolic diseases, though the underlying mechanisms remain unclear. Using a Wistar rat model of undernutrition over 50 generations, we show that Undernourished rats exhibit low birth-weight, high visceral adiposity (DXA/MRI), and insulin resistance (hyperinsulinemic-euglycemic clamps), compared to age-/gender-matched control rats. Undernourished rats also have higher circulating insulin, homocysteine, endotoxin and leptin levels, lower adiponectin, vitamin B12 and folate levels, and an 8-fold increased susceptibility to Streptozotocin-induced diabetes compared to control rats. Importantly, these metabolic abnormalities are not reversed after two generations of unrestricted access to commercial chow (nutrient recuperation). Altered epigenetic signatures in insulin-2 gene promoter region of Undernourished rats are not reversed by nutrient recuperation, and may contribute to the persistent detrimental metabolic profiles in similar multigenerational undernourished human populations.

INTRODUCTION

The burden of type 2 diabetes mellitus (T2D) is increasing worldwide, particularly in developing countries, where >70% of the global burden of T2D is predicted to exist by 2030 (Echouffo-Tcheugui and Dagogo-Jack, 2012). Although reasons for the increasing rates of T2D in developing countries are not fully elucidated, important factors include lifestyle changes involving rural-to-urban migration (“urbanization”), intra-uterine undernutrition, and fetal programming.

During the past two decades, increasing evidence arising from multiple clinical studies conducted by the research teams of Yajnik and Barker support an important role of early life undernutrition, and specifically disturbances of one-carbon metabolism, in the heightened susceptibility of (Asian) Indians to T2D at a younger age, and in the absence of generalized obesity (Yajnik et al., 1995, 2003, 2014; Yajnik and Deshmukh, 2012). These studies have highlighted body composition and nutritional-metabolic peculiarities of multigenerationally undernourished Indians: a thin-fat (low lean mass, high fat mass) phenotype compared to Europeans, with predominant visceral deposition of fat. This body composition is strongly associated with insulin resistance and related metabolic-endocrine abnormalities. Importantly, this “thin-fat” phenotype was present at birth and, therefore, programmed during intrauterine life, possibly through epigenetic mechanisms over multiple generations. Maternal intergenerational undernutrition, evident in stunting, low BMI, and a disturbance of dietary methyl donors (low protein and vitamin B12 and high folate status, related to vegetarian diets) appear contributory to the increased risk of diabetes and CVD in Indians (Yajnik, 2004; Yajnik and Deshmukh, 2012; Yajnik et al., 2003, 2008).

It is now well appreciated that the intra-uterine environment can induce heritable alterations that may be retained over generations (Aiken and Ozanne, 2014; Goodspeed et al., 2015; Ng et al., 2010). In non-human primates, a maternal high-fat diet supplemented with calorically dense treats leading to obesity...
has been shown to epigenetically alter chromatin structure in their progeny via SIRT1-mediated covalent modifications of histones (Aagaard-Tillery et al., 2008; Suter et al., 2012).

Increased adiposity and insulin resistance have also been reported in high-fat diet-fed rodent models. Intra-uterine programming may involve epigenetic changes, which can be passed over generations, and may promote the development of adiposity and T2D.

In a preliminary study of naturally occurring food-deprived (for 12 years) Wistar rats, we identified differences in body composition and defects in glucose-insulin metabolism. We therefore decided to study the above phenotype and underlying mechanisms by replicating the diets in this prospective hypothesis-driven study. We present herein the first direct evidence that Wistar rats that are protein calorically undernourished over multiple (50) generations show increased adiposity, insulin resistance, and susceptibility to Streptozotocin (STZ)-induced diabetes. We further demonstrate that this adverse metabolic state is associated with altered histone modification profiles, which cannot be reversed by two generations of nutrient recuperation.

RESULTS AND DISCUSSION

A Multigenerational Rat Model of Undernutrition

Wistar rats were maintained for 50 generations (Figure 1A; Figure S1A) with unrestricted access to standard commercial chow (“Control”) or restricted to 50% of ad libitum mass of a chow containing 2.2-fold less protein, 1.3-fold more carbohydrates, 2.1-fold less fat, and 2.4-fold less fiber (Tables S1A, and S1B) with low vitamin supplementation (Table S1C), as compared to Control chow. The Undernourished (U) rats were lighter than Control (C) rats (Figures 1B and 1C), had low birth weight (Figure 1D), and did not show any catchup growth (Figure 1C). Dual-energy X-ray absorptiometry (DXA) measurements demonstrated that Undernourished rats had less body fat (normalized to body weight) than Control rats at 12 weeks of age but increased and exceeded control levels significantly at 33 and 86 weeks of age (Figure 1E). Their bone mineral density (BMD) was lower than Control rats at all times (Figure 1F). Biometric assessment demonstrated increase in skin-fold thickness, abdominal girth, and BMI following multigenerational undernutrition (Figure 1G). Thus, undernutrition over 50 generations led to a phenotype that was lighter at birth, failed to show catchup growth, and demonstrated increasing adiposity later in life.

Attempting to Correct Metabolic Effects of Multigenerational Undernutrition

After 50 generations of undernutrition, Undernourished rats were provided with unrestricted access to a standard (Control) chow diet from day 0 of pregnancy, and their progeny were studied at second generation of recuperation (R2 rats). These rats (Figure 2A) showed restoration of birth weight (Figure 1D), and did not show any catchup growth (Figure 1C). Dual-energy X-ray absorptiometry (DXA) measurements demonstrated that Undernourished rats had less body fat (normalized to body weight) than Control rats at 12 weeks of age but increased and exceeded control levels significantly at 33 and 86 weeks of age (Figure 1E). Their bone mineral density (BMD) was lower than Control rats at all times (Figure 1F). Biometric assessment demonstrated increase in skin-fold thickness, abdominal girth, and BMI following multigenerational undernutrition (Figure 1G). Thus, undernutrition over 50 generations led to a phenotype that was lighter at birth, failed to show catchup growth, and demonstrated increasing adiposity later in life.

Figure 1. Generation of a Multigenerational Undernourished Rat

(A) Study design illustrating the period of undernutrition and nutrient transition (Recuperation). (B–F) (B) Control (C) and Undernourished (U) rats; bar, 10 cm, (C) growth curves of Control and multigenerational Undernourished rats showing low birth-weight (D) and no catch-up growth (E), (E) body fat and (F) bone mineral density measured using DXA at 12, 33, or 86 weeks. (G) biometric measurements in Control and Undernourished rats; n ≥ 8, >4 litters, data presented as mean ± SEM, *p < 0.05, **p < 0.01, ***p < 0.01, and ****p < 0.0001; all comparisons against Controls.
and 2B) after the post-weaning period (Figure 2B; Figures S1D and S1E). Recuperation (R2) rats had higher abdominal girth on day 4 (Figure S1F) and the highest fat mass of the three groups (Control, Undernourished, and Recuperation) at 12 and 33 weeks of age (Figure S1G). MRI identified increased fat deposition in visceral organs of R2 rats, especially the liver (Figure 2C; Movie S1). DXA measurements confirmed significantly higher body fat in Undernourished and R2 rats than Controls (Figure 2D), most of which was due to visceral adiposity (Figure 2E). Multigenerational undernutrition thus appears to support mechanisms favoring accumulation of body fat (Stewart et al., 1980; Wells, 2006) as an adaptive mechanism (the so-called “Thrifty phenotype” hypothesis). However, the adaptive mechanisms of the Undernourished rats were not suited to the changing (Recuperation) environment of unrestricted access to Control chow. Recuperation rats showed restoration of birth weight but heavier body mass, a lighter heart and pancreas, and a heavier liver and spleen compared with Control rats (Figure 2F). Increased hepatic weight was mostly a result of fat accumulation (Figures S2A and S2B), which may contribute to increased splenic weight (Francque et al., 2011; Murray et al., 1986). Brain weight (data not shown) was similar to that in Control rats (both genders). Undernourished rats had smaller muscle mass. Interestingly, the gut length was shorter in Undernourished rats and remained shorter in R2 rats. Protein-deprivation in rats has been shown to lead to shorter intestines (Kasai et al., 2012). Similar changes induced over multiple generations of undernutrition in our study appear to introduce heritable alterations that were not reversed after two generations of “normal” diet. No gender differences were observed (Figures S1B–S1E, S2B, S2G–S2J, S4A, and S4B).

**Unrestricted Access to a Control Diet in Multigenerational Undernourished Rats Promotes Adverse Metabolic Health in Later Life**

We hypothesized that Undernourished rats provided with unrestricted access to Control commercial chow would present with metabolic profiles that are comparable to Control rats. Undernourished rats (relative to Controls) demonstrated similar circulating concentrations of serum endotoxin (Table S1D) but higher concentrations of circulating glucose (p ≤ 0.01), insulin
Higher levels of SGPT in Undernourished and R2 rats compared to Control rats was consistent with liver damage due to fat deposition. We also observed that low levels of circulating vitamin B12 and folate in Undernourished rats were partially corrected in R2 rats. Macro-nutrient sufficiency thus seems to offer a considerable correction for vitamin B12 and folate deficiency as seen in the R2 rats, yet their levels remained significantly lower than Controls. Serum total homocysteine was elevated in Undernourished rats (versus Control) and did not reverse in R2 rats. Recuperation rats were visibly obese (Figure 2A) and showed sedentary habits as compared to Control rats (Movies S2 and S3), despite having similar total energy intake (Table S1B). Higher circulating leptin and lower adiponectin (Table S1D) levels in Undernourished and R2 rats reflected increased adiposity in these rats (Figures 1E and 2D; Figures S1F and S1G). Serum endotoxin concentrations were significantly higher in the R2 rats (Table S1D), as seen in human studies of obese, IGT, and T2D subjects (Harte et al., 2012). Similar to findings in mouse studies (Smith et al., 1966), elevations in serum endotoxin levels, along with hepatic fat (discussed above), may contribute to heavier spleens (Figure 2F) in Undernourished and R2 rats.

Fasting hyperinsulinemia was a prominent feature of Undernourished and R2 rats although islet insulin content was ~3-fold lower in Undernourished, but not R2, rats (Table S1D). We observed significant increases in numbers of insulin-containing cells in Undernourished and R2 rats with relatively fewer glucagon-containing cells (Figures S2C–S2E), though no significant increases in beta cell mass (Figure S2F) were observed.

Altered Metabolic Health following Multigenerational Undernutrition Is Not Restored through Macronutrient Supplementation

Following assessment of impaired glucose tolerance (Figures 3A and 3B; Figures S2G and S2H), hyperinsulinemic-euglycemic clamp studies were performed on Control, Undernourished, and R2 rats (Figures 3C and 3D; Figures S2I–S2J) to confirm insulin resistance. Significantly lower glucose infusion rates supported maintenance of clamped glucose concentrations in the Undernourished and R2 rats, confirming that the insulin resistance observed in the Undernourished rats was not restored following two generations of Control diet restoration. To understand whether undernutrition over generations altered the susceptibility to diabetes, we carried out a streptozotocin (STZ) dose response (see Experimental Procedures, Figure S3A). STZ, a pancreatic beta cell toxin, is routinely used to induce diabetes in Wistar rats. Undernourished rats died

Figure 3. Insulin Sensitivity and Susceptibility to STZ-Induced Diabetes

(A–D) (A) Glucose tolerance test, (B) insulin tolerance test, (C) glucose was clamped during hyperinsulinemic-euglycemic clamp, and (D) glucose infusion rate (GIR) was measured during the clamp.

(E–G) Survival curves for Streptozotocin (STZ) dose response in (E) Control, (F) Undernourished, and (G) R2 rats.

(H) Circulating insulin after STZ injection (200 mg/kg). Data presented as mean ± SD, n = 6 (4–12 litters). (A, B, and E–H) 14- to 20-week-old rats; (C and D) 33-week-old rats; *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001; all comparisons against Controls.
following exposure to a dose of STZ (200 mg/kg b.w.) that rendered ≥90% Control rats (Figure 3E) diabetic (fasting blood glucose > 11 mmol/l by day 8 post-STZ). An 8-fold lower dose (25 mg/kg b.w.) offered 100% survival in Undernourished (Figure 3F) as well as R2 rats (Figure 3G) but all developing diabetes with fasting blood glucose > 11 mmol/l by day 8 from STZ injection; none of the Control animals became diabetic at this (low) dose. We observed that Undernourished and R2 rats injected with this high dose (200 mg/kg b.w.) of STZ died with hypoglycemic convulsions in 12–14 hr (Figures 3F and 3G).

Serial circulating insulin measurements following STZ injection (200 mg/kg b.w.) in Undernourished and R2 rats (Figure 3H) demonstrated that the increased mortality at 200 mg/kg dose was indeed associated with a significant increase in circulating insulin within 3–6 hr after STZ injection, which resulted in hypoglycemic convulsions and death. As Undernourished and R2 rats showed 100% survival with fasting plasma glucose > 11 mmol/l by day 8, at a dose that is eight times lower than the diabetogenic dose in Control rats (200 mg/kg b.w.), Undernourished and R2 rats had eight times more susceptibility to STZ-induced diabetes.

Undernourished rats also showed other markers of metabolic disorder. Elevated levels of circulating tHcy (Table S1D) is related to higher risk of coronary disease, stroke, and peripheral vascular disease and atherosclerosis in man (Zhou and Austin, 2009). Electrocardiograms (Figures S3B–S3F) revealed inverted P and T waves in R2 rats, with elevated Q and ST-segments, consistent with myocardial infarction and associated with higher early mortality and morbidity (Anderson et al., 2007), in man. A lower circulating concentration of folate (Table S1D) may itself be an atherogenic factor (Imamura et al., 2010) that could promote hyperhomocysteinemia seen in these Undernourished and R2 rats. Cardiac histology revealed multiple morphological abnormalities in R2 rats (Figure S3G) and higher cardiac tissue levels of the DNA methyl transferase dnmt3a1 in Undernourished and R2 rats (Figure S3H), which may be associated with epigenetic silencing in cardiac tissue as well (Kotini et al., 2011).

Undernourished and R2 rat pancreas contained significantly fewer (pro-)insulin 2 gene transcripts (Figure 4A; Figures S4A and S4B), indicating that multigenerational undernutrition affected insulin gene transcription with no recovery. This may be a result of epigenetic repression of insulin gene transcription,
although active degradation of insulin gene transcripts, which have a long half-life (Gershengorn et al., 2004) of ~30–36 hr (in man), or both, is also a possibility. Indeed, the relative abundance of KMT1A, a histone-3 lysine 9-specific methyl transferase, which trimethylates H3K9me leading to suppression of gene transcription (Krauss, 2008; Rai et al., 2006), was increased in Undernourished and R2 pancreas (Figures S4C and S4D). To test whether the pro-insulin gene was epigenetically modified, we carried out chromatin immunoprecipitation (ChIP) for five different histone modifications: H3Ac, H4Ac, and H3K4me3, three modifications associated with transcriptionally activated gene promoters, and H3K9me3 and H3K20me3, two modifications associated with suppressed/silenced gene promoters. TaqMan-based real-time PCR was carried out on immunoprecipitated DNA to quantify the insulin promoter content in each of the IP fractions. Data comparing Undernourished and R2 islets to Control islets (Figure S4E) were logarithmically transformed to decrease in transcription factor PDX1 binding at the insulin-2 gene promoter. Overall, differences in chromosomal conformation induced as a result of these modifications led to significant decrease in transcription factor PDX1 binding at the insulin-2 gene promoter. All of these may contribute to altered gene expression observed in the Undernourished rats (Figure S4M). Intriguingly, nutrient recuperation for two generations did not reverse these epigenetic modifications, but rather led to increased obesity and metabolic risk for diabetes with electrocardiographic and histological evidence for cardiovascular disease.

Current investigations failed to show any associations with genetic polymorphisms (data not shown), but further studies are warranted. Our studies have largely focused on assessing the metabolic and epigenetic changes following multigenerational undernutrition and nutrient recuperation. The thrifty genotype hypothesis (Neel, 1999) proposed that increasing prevalence of T2D among populations undergoing nutrient/lifestyle transition resulted from the selection of metabolically thrifty genes. We questioned whether genetic factors are altered during multigenerational undernutrition and whether such changes are reversed by nutrient recuperation. We initiated targeted genetic analyses in the three rat populations. Sequencing of potential SNPs in mthfr and tcn2 genes (associated with cardiac, neural tube, and vitamin B₁₂ defects) as well as RNA-sequencing for Ins-2 transcripts showed no genetic polymorphisms at these loci (data not shown). Future studies involving a desired (40×) coverage through whole-genome sequencing will identify possible contributions of genetic polymorphisms toward metabolic health. Another limitation of the study is that we have assessed metabolic and epigenetic changes following multigenerational undernutrition and relatively short-term (two-generation) nutrient recuperation. Whether nutrient restoration to Undernourished animals for multiple (>2) generations may reverse adverse metabolic effects remains unknown. Another component that would be also interesting to understand is the effect of high-fat diet on Undernourished animals, which would mimic nutrient transition in today’s developing countries more accurately.

Additionally, studies involving metagenome sequencing, whole-genome sequencing, and epigenetic profiling in Undernourished, Recuperation, and Control rats during nutrient transition with micronutrient (vitamin B₁₂, folate, vitamin B₆, magnesium, and vitamin D) supplementation may identify instructive mechanisms that modify our epigenomes during adaptation to a changing diet and lifestyle. The Undernourished
glucose and insulin estimations were carried as detailed in Supplemental Experimental Procedures. From 4 to 12 different litters. (Abbott Laboratories) as detailed in Supplemental Experimental Procedures. of this outbred colony (Figure S1A). Data represent analyses on >6 animals this study were approved by the NCCS/NTC Ethics and Animal Welfare Committees. At least 20 litters were used at each generation for propagation of this outbred colony (Figure S1A). Data represent analyses on >6 animals from 4 to 12 different litters.

**Experimental Procedures**

Animals

Undernourished rats were derived from a colony of Wistar rats (Control) by feeding a protein caloric-deficient diet (Tables S1A–S1C), as outlined in the study design (Figure 1A). Animals were housed under 12 hr day/night cycle; Control and Recuperation rats were allowed free access to standard commercial chow and water at all times. National and Institutional guidelines for the use and care of laboratory animals were followed. All procedures detailed in this study were approved by the NCCS/NTC Ethics and Animal Welfare Committees. At least 20 litters were used at each generation for propagation of this outbred colony (Figure S1A). Data represent analyses on >6 animals from 4 to 12 different litters.

Biochemical Estimations

Glucose and insulin estimations were carried as detailed in Supplemental Experimental Procedures. Circulating biomarkers were measured on a Spectrum II Auto analyzer (Abbott Laboratories) as detailed in Supplemental Experimental Procedures.

Dual-energy X-ray absorptiometry (DXA) was carried out on age-matched males at 12, 33, or 86 weeks using Orthometrics pDEXA scanner. Total/visceral/s.c. fat mass were measured and adiposity were calculated as amount of fat normalized to body weight at the time of measurement.

MRT was performed on age-matched rats using a Siemens 1.5 Tesla machine with 3 mm sections.

Hyperinsulinemic-euglycemic clamp studies were carried out based on the guidelines and procedures detailed by Ayala et al. (2006).

Streptozotocin (STZ), a pancreatic β-cell toxin, was reconstituted in chilled citrate buffer (pH = 4.5) prior to l.p. injection and post-STZ survival was measured as detailed in Supplemental Experimental Procedures.

Immunostaining and confocal microscopy was carried out using methods detailed in Supplemental Experimental Procedures and published earlier (Joglekar et al., 2009).

ChIP and western blotting for epigenetic modulators was carried out as detailed in Supplemental Experimental Procedures.

Quantitative real-time PCR was carried out using SybrG or TaqMan assays as detailed in Supplemental Experimental Procedures. Data are presented as “Fold over detectable” as explained elsewhere (Hardikar et al., 2014).

**Statistical Analysis**

Differences between groups were calculated by using one-way or two-way ANOVA and appropriate post hoc tests as described in Supplemental Experimental Procedures. SPPS, GraphPad Prism, and Jandel Scientific softwares were used to assess/plot data. A sample size of six rats in each group is sufficient to identify a difference of 27% (SD 15% of mean), with 80% power at 2p = 0.05 between any two groups, or to identify a difference of 45% (SD 15% of mean), with 80% power at 2p = 0.05 between any two groups.

Statistical analysis was performed using Graphpad Prism software. Radar plots of the data representing ChIP studies were created by logarithmically transform- ing r fold over detectable data, using shoelace formula.

**Supplemental Information**

Supplemental Information includes Supplemental Experimental Procedures, four figures, one table, and three movies and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2015.06.008.

**Author Contributions**

A.A.H. designed, planned, carried cellular and molecular assays, data analyses, and wrote/revised the paper; S.N.S. performed animal work and biochemical assays, M.S.K. performed animal physiology studies; M.V.J. performed all epigenetic studies, immunostaining, and morphometry; W.W. and A.L. performed epigenetic studies; A.S.P. and S.K. performed clamps, DXA, and EKGs; D.S.B. performed biochemistry; A.J. performed statistics; M.R.U. conducted animal studies; A.K.R. and P.Y. performed molecular studies; R.R.B., K.A., S.G., A.C.K., A.J.J., and C.S.Y. provided infrastructure support, data analysis, and statistics. All authors read and contributed to modifications/revisions in final draft.

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