Dietary Protein to Carbohydrate Ratio and Caloric Restriction: Comparing Metabolic Outcomes in Mice

Graphical Abstract

Highlights

- Ad libitum low-protein, high-carbohydrate diets (LPHC) improve metabolic health

- Caloric restriction combined with LPHC diet does not provide added health benefits

- Energy intake and energy expenditure are increased on LPHC diets

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In Brief

Nutritional interventions improve metabolic health in mice. Solon-Biet et al. find that short-term ad libitum low-protein, high-carbohydrate (LPHC) diets improve levels of insulin, glucose, lipids, and HOMA. LPHC diets under ad-libitum-fed conditions generate the metabolic benefits of caloric restriction without a 40% reduction in total caloric intake.
Dietary Protein to Carbohydrate Ratio and Caloric Restriction: Comparing Metabolic Outcomes in Mice

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SUMMARY

Both caloric restriction (CR) and low-protein, high-carbohydrate (LPHC) ad-libitum-fed diets increase lifespan and improve metabolic parameters such as insulin, glucose, and blood lipids. Severe CR, however, is unsustainable for most people; therefore, it is important to determine whether manipulating macronutrient ratios in ad-libitum-fed conditions can generate similar health outcomes. We present the results of a short-term (8 week) dietary manipulation on metabolic outcomes in mice. We compared three diets varying in protein to carbohydrate ratio under both CR and ad libitum conditions. Ad libitum LPHC diets delivered similar benefits to CR in terms of levels of insulin, glucose, lipids, and HOMA, despite increased energy intake. CR on LPHC diets did not provide additional benefits relative to ad libitum LPHC. We show that LPHC diets under ad-libitum-fed conditions generate the metabolic benefits of CR without a 40% reduction in total caloric intake.

INTRODUCTION

Caloric restriction (CR) of ~30%–50% increases healthspan, delays the onset of aging and age-associated diseases, and improves metabolic health in most species (Everitt et al., 2010; Masoro, 2005; Mattison et al., 2012; McCay et al., 1935; Mercken et al., 2012; Weindruch et al., 1986). It is generally thought that CR is mediated directly by the reduction in energy intake impacting on cellular substrates such as NAD+ and AMP, with subsequent downstream effects on nutrient-sensing pathways such as sirtuin (SIRT1), AMP-activated protein kinase (AMPK), mechanistic target of rapamycin (mTOR), and insulin/insulin growth factor 1 (IGF-1) (Brunet et al., 2004; Fontana et al., 2010; Le Couteur et al., 2012). Although beneficial, CR is unsustainable in the vast majority of humans (Fontana and Partridge, 2015).

More recently, it has been demonstrated in studies using nutritional geometry that the balance of macronutrients has a profound impact on healthspan and lifespan in animals with ad libitum (AL) access to food (Lee et al., 2008; Piper et al., 2011; Solon-Biet et al., 2014). In these studies, CR induced by dietary dilution did not increase lifespan (Solon-Biet et al., 2014). In AL-fed mice and Drosophila melanogaster, diets low in protein and high in carbohydrates (LPHC) maximized lifespan, while a reduction of total energy intake had no positive impact on longevity (Lee et al., 2008; Solon-Biet et al., 2014). Moreover in mice, LPHC diets were associated with improved late-life cardiometabolic health (Solon-Biet et al., 2014) and a younger immune profile (Le Couteur et al., 2014). Low protein intake has also been associated with better health and reduced mortality in observational studies of humans (Levine et al., 2014), while high-protein, low-carbohydrate (HPLC) diets are associated with higher mortality, cardiovascular disease, and diabetes mellitus (Fontana and Partridge, 2015; Fung et al., 2010; Lagiou et al., 2012; Simpson et al., 2015).

Thus, a diet with altered macronutrient composition may be a more feasible intervention than severe CR for managing metabolic health in humans. However, there is a downside: whereas LPHC diets have beneficial effects later in life, they are associated with increased food intake, driven by compensatory feeding for protein (Gosby et al., 2011; Huang et al., 2013; Raubenheimer et al., 2015; Simpson and Raubenheimer, 2005). The clinical consequences of overconsumption are well established, including obesity, metabolic syndrome, type 2 diabetes mellitus, and fatty liver (Dietrich and Hellerbrand, 2014; Nseir et al., 2014; Simpson et al., 2015). Overall, reducing food intake and body weight improves the manifestations of metabolic syndrome and fatty liver (Ajala et al., 2013; Nseir et al., 2014). Effects of macronutrients on these outcomes in humans are less clear, but, in general, high-carbohydrate diets are thought to contribute to fatty liver and metabolic syndrome, while high-protein diets might be protective (Nseir et al., 2014), in part through aiding reduced energy intake (Gosby et al., 2014).

The question arises as to which dietary intervention is more effective at improving metabolic health and whether there is any synergy between these dietary regimens. In this study, we directly compared CR with diets differing in protein to
carbohydrate ratio and evaluated several metabolic and hepatic outcomes. The results indicate that LPHC diets under AL-feeding conditions achieve the metabolic gains seen with CR. As expected, LPHC was associated with increased energy intake, but over a period of 8 weeks this was counterbalanced by increased energy expenditure and did not lead to a significant increase in body adiposity or fatty liver. Additional health improvements were not achieved by combining CR with LPHC diets, although CR prevented the negative metabolic consequences of a HPLC diet in AL-fed mice.

RESULTS

Food and Energy Intake
Dietary protein to carbohydrate balance (P:C) influenced food and energy intakes in AL-fed animals (Figures 1A and 1B). AL mice titrated their food intake according to percent dietary protein, with animals on AL LPHC diet consuming the greatest amounts of food and energy. After the 8-week dietary intervention, CR animals had reduced body mass compared with AL-fed animals, while mice on the AL medium P:C (MPMC) diets had the highest body mass (Figure 1C).

Liver and Pancreatic Pathology
All diets were associated with normal liver histology regardless of whether mice were fed AL or CR (Figures 3A and 3B). There were subtle changes in the porosity of the liver sinusoidal endothelium, with lower porosity observed in LPHC compared with MPMC or HPLC mice (1.13±0.11% versus 1.63±0.12%, p = 0.02; Figures 3C and 3D). There were no obvious changes in pancreatic islet pathology or pancreatic insulin stains (χ² = 2.33, df = 5, p = 0.8; Figure S2), but there was an increase in the intensity of staining for glucagon in AL HPLC mice compared to all other groups (χ² = 26.09, df = 5, p ≤ 0.0001; Figures 3E–3G). This suggests the AL HPLC diet results in increased glucagon secretion, causing elevated blood glucose levels, and glucose intolerance. The higher insulin and HOMA levels observed (Figures 2A and 2B) are consistent with this notion.

Energy Expenditure, RER, and Body Composition
Energy expenditure was significantly higher in the AL LPHC animals compared to all of the CR animals. The respiratory exchange ratio (RER) approached 1 in the AL LPHC animals, indicating carbohydrate was the primary source of energy, compared to the other groups, where the value approached 0.7, indicating utilization of fat (Figure 4A; Table S2). There were discernible effects of dietary interventions on body composition. Body mass was lower in the CR animals (Figure 1C) while, interestingly, percentage body fat showed opposing patterns, with animals in CR groups tending to have increased adiposity (relative to lean mass) (Figure 4C).

DISCUSSION

Our results provide a direct comparison of CR to AL LPHC diets, to determine whether it is possible to generate similar metabolic outcomes achieved with CR using AL diets. Our results show that, after 8 weeks, AL-fed LPHC mice had similar metabolic improvements as seen under CR, despite increased energy intake, but without the development of increased body adiposity and fatty liver that is observed in longer-term chronic LPHC feeding. Manipulating dietary P:C ratios in animals under CR conditions did not generate any additional benefits in terms of these outcomes, nor did it cause any detrimental effects to the mice.

Mice, like humans and various other species, demonstrate “protein leverage,” where protein intake is prioritized over fat and carbohydrates ( Gosby et al., 2011; Raubenheimer et al., 2015; Simpson and Raubenheimer, 2005 ). Such an effect was evident in the present study, with the AL LPHC diet resulting in compared with the other AL diets, and their results are comparable to those found in CR-fed animals. These same outcomes were also improved to a similar extent in all CR treatments, regardless of dietary P:C (Table S2). Triglycerides followed a similar pattern, with highest levels in the animals on the AL HPLC and AL MPMC diets, while there were no significant differences between the AL LPHC diets and any of the CR diets. A similar trend was seen for HDLc where the lowest (worse) values were seen for the AL HPLC and AL MPMC diets.
increased food and energy intake of about 25%–30% compared to the AL HPLC diet. Despite this elevated intake, we did not observe increased adiposity, body weight, or diet-induced fatty liver in AL LPHC mice. They did, however, show increased energy expenditure, which is consistent with increased diet-induced thermogenesis (DIT) serving to dissipate excess ingested energy and slow development of adiposity (Huang et al., 2013; Stock, 1999).

Exposure to LPHC diets over longer time periods, however, has been associated with increased body weight, adiposity, and fatty liver (Huang et al., 2013; Solon-Biet et al., 2014; Sørensen et al., 2008), indicating that mechanisms for compensatory energy expenditure may become progressively less effective with time (Huang et al., 2013). Such mice, albeit more adipose, nonetheless have improved cardiometabolic outcomes, including insulin, GTT, HOMA, lipids, and blood pressure (Solon-Biet et al., 2014). It is also important to consider the type of carbohydrate consumed (e.g., starch versus fructose versus glucose [Wylie-Rosett et al., 2004]) as this has been shown in rodent studies to have a profound influence on the development of obesity and insulin resistance (Maki and Phillips, 2015; Storlien et al., 1988; Thorburn et al., 1989; Thresher et al., 2000) and thus may have a considerable effect on cardiometabolic health if fructose is a significant component of an LPHC diet.

AL HPLC diets were associated with decreased insulin sensitivity, indicated by elevated circulating insulin, HOMA, and pancreatic glucagon. This metabolic dysregulation may be attributed to the upregulation of gluconeogenesis, subsequently increasing glycogen turnover and total hepatic glucose output (Eisenstein et al., 1974; Linn et al., 2000). Whereas HPLC diets do not sustain optimal late-life cardiometabolic health, it is important to note that nutritional requirements change with age, and higher P:C diets are required to support reproduction rather than sustain maximal lifespan (Simpson et al., 2015; Solon-Biet et al., 2014, 2015).

Here, we have compared the metabolic effects of short-term CR and AL LPHC diets in mice. The results of this study suggest that it may be possible to titrate the balance of macronutrients to gain some of the metabolic benefits of CR, without the challenge of a 40% reduction in caloric intake. A central priority is to further investigate and compare the long-term effects of traditional CR and AL LPHC diets on metabolic health and lifespan in mice and other model organisms, as well as to begin to consider the effects of the type and quality of proteins and carbohydrates.

**EXPERIMENTAL PROCEDURES**

**Animals and Dietary Interventions**

Male C57BL6/J mice (3 weeks old; n = 90; Jackson Laboratory) were housed in groups of five at the National Institute of Aging. Animals were kept at 22°C under a 12:12-hr light-dark cycle (12-hr dark period starting at 18:00), and were micro-chipped (Biomedic Data Systems) for individual identification and temperature quantification. All animal protocols were approved by the Gerontology Research Center Animal Care and Use Committee (352-LEG-2012) of the National Institute on Aging.
Three experimental diets were formulated that differed in protein to carbohydrate ratios based on Solon-Biet et al. (2014). These diets were classified as low protein, high carbohydrate (5% protein; LPHC); medium protein, medium carbohydrate (33% protein; MPMC); and high protein, low carbohydrate (60% protein, HPLC). Fat was fixed at 20% of total energy for all three diets. Experimental diets were isocaloric (4 kcal/g) and contained the same ingredients (Table S1). All diets were manufactured in dry pelleted form by Dyets. Mice were assigned to one of six different dietary regimens: ad libitum access to diets where the protein to carbohydrate ratio was either high (AL HPLC), medium (AL MPMC), or low (AL LPHC), and caloric restricted access to diets where the protein to carbohydrate ratio was either high (CR HPLC), medium (CR MPMC), or low (CR LPHC).

At 8 weeks of age, mice underwent a 4-day acclimatization period of a 50/50 food combination of standard chow and experimental diet, followed by solely experimental diets for the remainder of the study. Mice were randomly assigned to either an AL- or a 40% CR-feeding regimen. Bi-weekly food measurements of AL animals were used to calculate daily portions for CR-fed mice, where food was reduced increments of 10% starting at 20% until mice reached 40% CR. AL animals were allowed free access to respective diets for 8 weeks and were fed in the hopper, while CR mice were fed daily at approximately 8 a.m. ± 1 hr, with pellets dropped onto the cage of the floor. Body weights and temperature were recorded bi-weekly and food intakes for all groups were quantified at the same time. After 8 weeks of feeding, mice were euthanized and blood and tissues were collected for histological and biochemical analyses. On the day of the sacrifice, CR mice were not fed while AL mice were allowed to eat normally.

**Body Composition**
Fat, lean, and fluid mass of mice were measured using nuclear magnetic resonance imaging (NMR) with the Minispec LF90 (Bruker Optics). Unanesthetized mice were weighed and then scanned.

**Glucose and Insulin**
Oral glucose tolerance tests (OGTTs) were performed after 8 weeks of experimental diets. Mice were fasted overnight (16 hr) prior to testing then gavaged with a 30% glucose solution (1.5 g kg\(^{-1}\) body weight) and blood glucose measurements recorded at 0, 15, 30, 60, and 120 min via tail snip using a handheld glucometer (Bayer). The incremental area under the curve was calculated using mean values per cage. Insulin was measured in fasting blood samples using an enzyme-linked immunosorbent assay (Crystal Chem). The homeostatic model assessment (HOMA; http://www.dtu.ox.ac.uk/homa), which reflects insulin resistance, was determined from the product of the fasting glucose and insulin.

**Metabolic Rate**
In order to estimate whole-animal metabolic rate, substrate utilization, and physical activity, eight animals per group were housed individually and assessed by indirect calorimetry in an open-circuit oxymax chamber (Comprehensive Lab Animal Monitory System, CLAMS; Columbus Instruments). Oxygen consumption (VO\(_2\)) and carbon dioxide production (VCO\(_2\)) were measured over 48 hr and maintained at 24°C under at 12:12-hr light-dark cycle. Mice were acclimatized to metabolic cage conditions for 8 hr prior to the start of data recording. The respiratory exchange ratio (RER) was calculated as a ratio of VCO\(_2\) produced/VO\(_2\) consumed. An RER of 0.7 indicates that fat is the predominant fuel source, while an RER closer to 1.0 indicates that carbohydrate is the primary fuel.

**Liver and Pancreatic Pathology**
Paraffin-embedded liver tissue was sectioned and stained with H&E. Embedded pancreas tissue was sectioned and probed with monoclonal anti-glucagon antibody (Sigma G2654), monoclonal anti-insulin antibody (Sigma I2018), and anti-mouse IgG1 produced in rabbit (Sigma 1213701171). The extent of fatty liver and glucagon staining intensity was assessed and scored (0, +, ++, ++++) by four independent observers blinded to the tissue category. Liver tissue was also needle-perfused with saline, followed by 3% glutaraldehyde/2% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.4), 2% (w/v) sucrose, and 2 mM CaCl\(_2\). Following post-fixation with osmium tetroxide, graded dehydration in ethanol and hexamethyldisilazane, 1-mm\(^3\) blocks of liver were sputter coated with platinum and examined using a JEOL 6380 scanning electron microscope (JEOL) at 20,000X magnification. Ten random images were taken per sample and fenestration diameter analyzed using ImageJ software (Coggar et al., 2015).

**Statistical Analysis**
Data are presented as mean ± SEM, and differences are considered significant when p < 0.05. Comparisons between feeding regimens and diets on various responses were analyzed using ANOVA and post hoc Fisher’s LSD tests when indicated. Fisher’s LSD test was used to test for differences between AL LPHC and CR diets. Two-group comparisons were performed using Student’s t tests and Mann-Whitney rank sum tests (SigmaPlot v.11.2.0.5, Systat Software).
Intensity of glucagon and insulin staining was compared using a chi-square test in Microsoft Excel.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.05.007.

AUTHOR CONTRIBUTIONS

S.M.S.-B. designed and performed the experiments, analyzed the data, and wrote the manuscript. S.J.M. performed the experiments. S.C.P.C. wrote the manuscript and contributed to data analysis. V.C.C. and R.G. performed the histology. A.C.M. assisted in preparation of histological samples. D.G.L.C. and S.J.S. supervised the project, analyzed the data, and wrote the manuscript.

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