PROTEIN

The Good, The Bad, and The Ugly

Ron Rosedale M.D.

The Good...

We cannot live without it

The Bad...

Misc problems with protein:
homocysteine?
Brain?

The effect of high-protein diets on coronary blood flow.

Angiology 2000 Oct;51(10):817-26 (ISSN: 0003-3197)

Fleming RM

The Fleming Heart and Health Institute and the Camelot Foundation, Omaha, Nebraska 68114, USA.

Recent research has demonstrated that successful simultaneous treatment of multiple risk factors including cholesterol, triglycerides, **homocysteine**, lipoprotein (a) [Lp(a)], fibrinogen, antioxidants, endothelial dysfunction, inflammation, infection, and dietary factors can lead to the regression of coronary artery disease and the recovery of viable myocardium. However, preliminary work revealed that a number of individuals enrolled in the original study went on popular high-**protein** diets in an effort to lose weight. Despite increasing numbers of individuals following high-**protein** diets, little or no information is currently available regarding the effect of these diets on coronary artery disease and coronary blood flow. Twenty-six people were studied for 1 year by using myocardial perfusion imaging (MPI), echocardiography (ECHO), and serial blood work to evaluate the extent of changes in regional coronary blood flow, regional wall motion abnormalities, and several independent variables known to be important in the development and progression of coronary artery disease. Treatment was based on **homocysteine**, Lp (a), C-reactive **protein** (C-RP), triglycerides, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, very low-density lipoprotein cholesterol, and fibrinogen levels. Each variable was independently treated as previously reported. MPI and ECHO were performed at the beginning and end of the study for each individual. The 16 people (treatment group/TG) studied modified their dietary **intake** as instructed. Ten additional individuals elected a different dietary regimen consisting of a "high-**protein**" (high **protein** group/HPG) diet, which they believed would "improve" their overall health. Patients in the TG demonstrated a reduction in each of the independent variables studied with regression in both the extent and severity of coronary artery disease (CAD) as quantitatively measured by MPI. Recovery of viable myocardium was seen in 43.75% of myocardial segments in these patients, documented with both MPI and ECHO evaluations. Individuals in the HPG showed worsening of their independent variables. Most notably, fibrinogen, Lp (a), and C-RP increased by an average of 14%, 106%, and 61% respectively. Progression of the extent and severity of CAD was documented in each of the vascular territories with an overall cumulative progression of 39.7%. The differences between progression and extension of disease in the HPG and the regression of disease in the TG were statistically ($p<0.001$) significant. Patients following recommended treatment for each of the independent variables were able to regress both the extent and severity of their coronary artery disease (CAD), as well as improve their myocardial wall motion (function) while following the prescribed medical and dietary guidelines. However, individuals receiving the same medical treatment but following a high-**protein** diet showed a worsening of independent risk factors, in addition to progression of CAD. These results would suggest that high-**protein** diets may precipitate progression of CAI) through increases in lipid deposition and inflammatory and coagulation pathways.

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High Protein reduces insulin sensitivity

J Clin Endocrinol Metab. 1996 Nov;81(11):3938-43.

Effect of dietary protein intake on insulin secretion and glucose metabolism in insulin-dependent diabetes mellitus.

Linn, T, Geter, R, Prassek, S, Laube, H

Diabetology and Metabolism Unit, Justus Liebig University, Giessen, Germany.

Adult-onset insulin dependent diabetes mellitus (IDDM) is associated with significant residual insulin secretion. The process leading to the ultimate destruction of B cells may be influenced, among other factors, by the quality and amount of ingested protein. Using a standardized food questionnaire, we matched 13 individuals with normal protein (NP; 0.74 +/- 0.08 g/kg.day) and high protein (HP; 1.87 +/- 0.26 g/kg.day) intake from a sample of 117 newly diagnosed IDDM patients according to sex, age, body mass index, and energy intake. Nondiabetic control subjects were also selected. Dietary habits did not change significantly over an observation period of 1 yr. Glucagon-stimulated C peptide was significantly higher in the NP compared to the HP group (0.71 +/- 0.06 vs. 0.50 +/- 0.04 nmol/L; P < 0.002). NP food was associated with higher overall insulin sensitivity in both patients and nondiabetic subjects. Hepatic glucose output was significantly increased in individuals with HP intake [HP IDDM, 14.8 +/- 0.6 vs. NP IDDM, 12.7 +/- 0.7 (P < 0.01); HP control, 12.2 +/- 0.5 vs. NP control, 10.9 +/- 0.5 (P < 0.01 mumol/kg.min). Insulin-mediated suppression of hepatic glucose production was impaired in diabetic patients with high protein intake, but not in patients with normal protein diet. Gluconeogenesis estimated from 13C enrichment in breath and plasma was increased in individuals on a HP diet. We conclude that a NP diet is accompanied by delayed progression of the continuous loss of endogenous insulin in IDDM. This phenomenon is possibly due to decreased insulin demand on the B cells and/or reduced hepatic glucose production favoring enhanced insulin sensitivity.

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Metabolism 1994 Apr;43(4):462-7

Effects of dietary protein restriction on glucose and insulin metabolism in normal and diabetic humans.

Lariviere F, Chiasson JL, Schiffrin A, Taveroff A, Hoffer LJ

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We determined whether the amount of protein in the diet can affect insulin requirements in subjects with diabetes mellitus and glucose metabolism in normal subjects. Seven normal-weight volunteers with uncomplicated, intensively controlled, type I (insulin-dependent) diabetes and 12 similar nondiabetic subjects were studied on a metabolic ward before and after consuming a maintenance-energy but protein-free diet for 10 days. Blood glucose levels of diabetic subjects were measured seven times daily in response to insulin administration by continuous subcutaneous infusion. The plasma glucose appearance rate (Ra) was measured in seven normal subjects and all diabetic subjects using a primed-continuous infusion of D-[6,6-2H2]glucose. After adaptation to the protein-restricted diet, diabetic subjects experienced a 30% decrease in average preprandial and average daily blood glucose concentrations ($P < .01$); this occurred despite a concurrent 25% decrease in both basal and bolus insulin dosages ($P < .001$). Protein restriction decreased the postabsorptive glucose Ra ($P < .05$) and insulin concentrations ($P < .01$) of normal subjects by 20%, and increased their fasting glucagon concentrations by 24% ($P < .01$). We conclude that severe protein restriction decreases insulin requirements in type I diabetes and fasting hepatic glucose output and basal insulin levels in normal subjects. This effect appears to be mediated in part by decreased hepatic gluconeogenesis, but a contributory influence of increased insulin sensitivity is not ruled out.

PMID: 8159104, UI: 94211139

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Protein increases levels of insulin and leptin;
short term benefit; long term ills

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Central nervous system control of food intake and body weight

G. J. Morton[1], D. E. Cummings[2], D. G. Baskin[2,3], G. S. Barsh[4] & M. W. Schwartz[1]

In an appetizing food testifies to the efficiency with which the central nervous system (CNS) processes information of surprising variety and complexity. With the aid of cognitive, visual and olfactory cues, food items must first be identified and distinguished from a nearly infinite array of potentially toxic environmental constituents. Using taste information, the food's palatability is then assessed and integrated with both short- and long-term signals regarding nutritional state. One consequence of this integration is that the drive to eat decreases as food is ingested (termed 'satiation'), ensuring that the amount consumed in a single meal does not exceed what the body can safely handle. Changing energy requirements is another factor that can influence food consumption. Through a process known as energy homeostasis, food intake is adjusted over time so as to promote stability in the amount of body fuel stored as fat. In this way, through diverse blood-borne and afferent neural signals, information regarding nutrient status and energy stores is communicated to the brain where it is integrated with cognitive, visual, olfactory and taste cues—all happening unconsciously, before the first bite is taken.

Here we describe CNS mechanisms that regulate food intake, and review evidence that in response to reduced body fat stores, adaptive changes occur in neuronal systems governing both food-seeking behaviour (important for meal initiation) and satiety perception (important for meal termination). The net effect is that in response to weight loss, both the motivation to find food and the size of individual meals tend to increase until energy stores are replenished (Fig. 1), and mutation of any of several key molecules involved in this process has been shown to cause severe obesity in both animal models and humans. Despite this progress, the many fundamental questions remaining unanswered represent rich opportunities for future study.

Energy homeostasis

Obesity, by definition, results from ingesting calories in excess of ongoing requirements. Although environmental and lifestyle factors contribute to obesity pathogenesis, homeostatic adaptations to weight loss induced by voluntary caloric restriction are robust in both lean and obese individuals. In addition, normal-weight individuals are protected against expansion of body fat stores induced by weight gain as well as weight loss, at least in normal-weight individuals. Together, these findings indicate that obesity involves the defence of an elevated body weight, rather than the absence of regulation, and that deleterious interactions between obesity-promoting environmental factors and homeostatic control systems contribute to common forms of obesity and, hence, the global obesity pandemic.

Adiposity negative feedback. Introduced more than 50 years ago, the 'adiposity negative-feedback' model of energy homeostasis is founded on the premise that circulating signals inform the brain of changes in body fat mass and that in response to this input, the brain mounts adaptive adjustments of energy balance to stabilize fat stores[3]. Proposed criteria for a negative-feedback signal include: (1) that it circulates at levels proportionate to body fat content and enters the brain; (2) that it promotes weight loss by acting on neuronal systems implicated in energy homeostasis; and (3) that blockade of these neuronal actions increases food intake and body weight. Although many nutrients (for example, free fatty acids and glucose), cytokines (for example, interleukin-6, tumour necrosis factor-α) and hormones (for example, glucocorticoids) fulfill some of these criteria, only leptin and insulin satisfy all of them[4].

Studies in primitive organisms such as the nematode, *Caenorhabditis elegans*, and the fruitfly, *Drosophila melanogaster*, implicate insulin as a key ancestral negative-feedback regulator of body fuel stores[5,6]. By comparison, leptin has not been detected in invertebrates and probably evolved more recently[7]. Although genetic and pharmacological studies[8,9] suggest a more critical role for leptin than insulin in mammalian energy homeostasis, cross-talk between these hormones with respect to both the neuronal subsets and signal transduction pathways on which they act offers evidence of their shared evolutionary past.

Although leptin administration causes weight loss in diverse mammalian species, enthusiasm surrounding leptin as a therapeutic agent diminished rapidly with the discovery that leptin resistance is common among obese individuals[10]. Because obesity has long been associated with insulin resistance in peripheral tissues, it is perhaps not surprising that in obese rats, the hypothalamus develops resistance to insulin[11] as well as leptin[12]. Although reduced neuronal signalling by either hormone induces hyperphagia and weight gain

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The role of leptin in leptin resistance and obesity.

Author:

Zhang Y , Scarpace PJ

Source:

Physiol Behav, 88(3): 249-56 2006

Abstract:

Although the presence of hyperleptinemia with leptin resistance and obesity has long been recognized, a causal role of elevated leptin in these biological states remains unclear. This article summarizes some recent work from our laboratory supporting the concept that leptin, in and of itself, promotes leptin resistance and such resistance compounds the metabolic impact of diet-induced obesity. Results from multiple studies demonstrate that (1) chronically elevated central leptin decreases hypothalamic leptin receptor expression and protein levels and impairs leptin signaling; (2) leptin resistance and obesity are associated with reduced leptin receptors and diminished maximal leptin signaling capacity; and (3) leptin resistance confers increased susceptibility to diet-induced obesity. In essence, the augmented leptin accompanying obesity contributes to leptin resistance, and this leptin resistance promotes further obesity, leading to a vicious cycle of escalating metabolic

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Dietary composition and physiologic adaptations to energy restriction

Michael SD Agus, Janis F Swain, Courtney L Larson, Elizabeth A Eckert, and David S Ludwig

Am J Clin Nutr 2000 Apr;71(4):901-7 (ISSN: 0002-9165)

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BACKGROUND: The concept of a body weight set point, determined predominantly by genetic mechanisms, has been proposed to explain the poor long-term results of conventional energy-restricted diets in the treatment of obesity. OBJECTIVE: The objective of this study was to examine whether **dietary** composition affects hormonal and metabolic adaptations to energy restriction. DESIGN: A randomized, crossover design was used to compare the effects of a high-glycemic-index (high-GI) and a low-glycemic-index (low-GI) energy-restricted **diet**. The macronutrient composition of the high-GI **diet** was (as percent of energy) 67% carbohydrate, 15% **protein**, and 18% fat and that of the low-GI **diet** was 43% carbohydrate, 27% **protein**, and 30% fat; the diets had similar total energy, energy density, and fiber contents. The subjects, 10 moderately overweight young men, were studied for 9 d on 2 separate occasions. On days -1 to 0, they consumed self-selected foods ad libitum. On days 1-6, they received an energy-restricted high- or low-GI **diet**. On days 7-8, the high- or low-GI diets were consumed ad libitum. RESULTS: Serum **leptin** decreased to a lesser extent from day 0 to day 6 with the high-GI **diet** than with the low-GI **diet**. Resting energy expenditure declined by 10.5% during the high-GI **diet** but by only 4.6% during the low-GI **diet** (7.38 +/- 0.39 and 7.78 +/- 0.36 MJ/d, respectively, on days 5-6; P = 0.04). Nitrogen balance tended to be more negative, and energy intake from snacks on days 7-8 was greater, with the high-GI than the low-GI **diet**. CONCLUSION: Diets with identical energy contents can have different effects on **leptin** concentrations, energy expenditure, voluntary food intake, and nitrogen balance, suggesting that the physiologic adaptations to energy restriction can be modified by **dietary** composition.

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Macronutrient composition of the diet differentially affects leptin and adiponutrin mRNA expression in response to meal feeding.

Author:
Polson DA , Thompson MP

Source:
J Nutr Biochem, 15(4): 242-6 2004

Abstract:
A number of adipose-specific genes, including adiponutrin and the adipocytokines, appear to be involved in regulating overall energy balance, as their expression is dysregulated in various obese states and is responsive to feeding. This study determined the effect of meal-feeding diets of markedly different macronutrient composition (70% by weight protein or fat) on the expression of adiponutrin and several adipocytokines in white adipose tissue of rats. Adiponutrin mRNA rapidly increased by at least 8-fold within 3 hours after the high-protein meal. This response was similar to that seen after a high-sucrose meal (70% by weight of sucrose). In contrast, leptin mRNA was unchanged after the high-protein meal, whereas it increased more than 5-fold after a high-sucrose meal. On the high-protein diet the leptin mRNA did not decline upon fasting after the meal, whereas on the high-sucrose diet fasting brought about a rapid decline in leptin mRNA, suggesting that the composition of the diet had altered mRNA turnover. In rats on diets high in either saturated or polyunsaturated fats, adiponutrin mRNA remained at fasting levels even after the meals. Leptin mRNA was unchanged and was maintained at post-meal levels. Resistin and acrp30/adiponectin mRNAs remained unchanged regardless of the macronutrient composition of the diet. The mechanism by which macronutrient composition of the diet is able to differentially influence the expression of adiponutrin and the adipocytokines, leptin, resistin, and acrp30/adiponectin remains to be determined.

Language:
eng

Macronutrient composition of the diet differentially affects leptin and adiponutrin mRNA expression in response to meal feeding.

Author:
Polson DA , Thompson MP

Source:
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adipocytokines in white adipose tissue of rats. Adiponutrin mRNA rapidly increased by at least 8-fold within 3 hours after the high-protein meal. This response was similar to that seen after a high-sucrose meal (70% by weight of sucrose). In contrast, leptin mRNA was unchanged after the high-protein meal, whereas it increased more than 5-fold after a high-sucrose meal. On the high-protein diet the leptin mRNA did not decline upon fasting after the meal, whereas on the high-sucrose diet fasting brought about a rapid decline in leptin mRNA, suggesting that the composition of the diet had altered mRNA turnover. In rats on diets high in either saturated or polyunsaturated fats, adiponutrin mRNA remained at fasting levels even after the meals. Leptin mRNA was unchanged and was maintained at post-meal levels. Resistin and acrp30/adiponectin mRNAs remained unchanged regardless of the macronutrient composition of the diet. The mechanism by which macronutrient composition of the diet is able to differentially influence the expression of adiponutrin and the adipocytokines, leptin, resistin, and acrp30/adiponectin remains to be determined.

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Am J Physiol Endocrinol Metab (March 1, 2005). doi:10.1152/ajpendo.00602.2004 1

REGULATION OF LEPTIN SECRETION FROM WHITE ADIPOCYTES BY INSULIN, GLYCOLYTIC SUBSTRATES AND AMINO ACIDS

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Running title: energy substrates in leptin secretion

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343-6111 p3094

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amino acids precursors of citric acid cycle intermediates potently stimulate *per se* basal leptin secretion, insulin having an additive effect

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Nutrient-sensing mTOR-mediated pathway regulates leptin production in isolated rat adipocytes

CECILIA ROH, JIANRONG HAN, ALEXANDROS TZATSOS, AND KONSTANTIN V. KANDROR
Boston University School of Medicine, Boston, Massachusetts 02118

leucine to isolated rat adipocytes significantly increased leptin production by these cells, suggesting that postprandial leptin levels may be directly regulated by dietary leucine. The effect of leucine was inhibited by rapamycin and not by actinomycin D. Besides, leucine administration did not increase the amount of leptin mRNA in adipocytes. Therefore, we concluded that leucine activates leptin expression in adipose cells at the level of translation via a mammalian target of rapamycin (mTOR)-mediated pathway. Because leptin is a secreted protein, its biosynthesis is compartmentalized on the endoplasmic reticulum. To analyze mTOR signaling in this subcellular fraction, we separated adipose cells by centrifugation into a heavy membrane fraction that includes virtually all endoplasmic reticulum and the cytosolic extract. Phosphorylation of the major mTOR targets, the ribosomal protein S6 and the translational inhibitor 4E-binding protein (BP)/phosphorylated heat- and acid-stable protein (PHAS)-1, was stimulated by leucine in the cytosolic extract, whereas, in the heavy fraction, S6 was constitutively phosphorylated and leucine only induced phosphorylation of 4E-BP/PHAS-1. We also found that 60–70% of leptin mRNA was stably associated with the heavy fraction, and leucine administration did not change the ratio between compartmentalized and free cytoplasmic leptin mRNA. We suggest that, in adipose cells, a predominant part of leptin mRNA is compartmentalized on the endoplasmic reticulum, and leucine activates translation of these messages via the mTOR/4E-BP/PHAS-1-mediated signaling pathway.

mammalian target of rapamycin

LEPTIN IS PRODUCED mainly by adipose cells and regulates food intake and whole body energy balance (36). Pursuant to this physiological role, circulating leptin levels rapidly increase after feeding (20) and decrease after food deprivation (9). Because leptin mRNA levels in adipose tissue also follow this pattern (3, 34), it has been generally accepted that leptin expression is controlled at the level of transcription (1). Although this tant to actinomycin D, and insulin administration does not change the amount of leptin mRNA in vitro (Ref. 4 and C. Roh and K. V. Kandror, unpublished observation). This suggests that insulin may not regulate or may not exclusively regulate the *ob* gene expression at the level of transcription.

Alternately, leptin expression may also be regulated at a posttranscriptional level. Mammalian cells possess an important nutrient-sensing pathway that controls protein synthesis at the level of translation. A central player in this pathway is a phosphatidylinositol kinase-related protein kinase called target of rapamycin (mTOR; see Refs. 27, 32, 35). mTOR is activated by free amino acids (13, 32), especially by leucine (23) via a mechanism that is yet unknown. mTOR stimulates translation of stored mRNAs through S6- and/or phosphorylated heat- and acid-stable protein (PHAS)/4E-binding protein (BP)-mediated pathways (27, 32, 35). Both pathways are readily activated by leucine in adipocytes (Refs. 7 and 8 and Fig. 3). We proposed that mTOR may be an appropriate nutrient sensor for leptin expression in adipose cells.

In agreement with this hypothesis, we found that addition of leucine to isolated rat adipocytes significantly stimulated leptin secretion in a rapamycin-sensitive and an actinomycin D-resistant fashion. Thus dietary leucine may increase leptin production via activation of mTOR and subsequent activation of leptin mRNA translation. This mechanism may provide a long-sought-after connection between food intake and leptin levels in blood.

MATERIALS AND METHODS

Antibodies. Affinity-purified polyclonal antibodies against phosphorylated S6 ($Ser^{235/236}$), p70 S6 kinase (Thr^{389}), and 4E-BP-1/PHAS-I (Ser^{65}) were from Cell Signaling (Beverly,

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Protein increases leptin

Triggers hexosamine pathway

...And the Ugly...

it appears that

High Protein Accelerates Aging

Journal of Gerontology: BIOLOGICAL SCIENCES
2005, Vol. 60A, No. 5, 549–555

Counting the Calories: The Role of Specific Nutrients in Extension of Life Span by Food Restriction

Matthew D. W. Piper, William Mair, and Linda Partridge

Department of Biology, University College London, United Kingdom.

Journal of Gerontology: BIOLOGICAL SCIENCES Copyright 2005 by The Gerontological Society of America
2005, Vol. 60A, No. 5, 549–555

as well as highlight the major advances already made. Delineation of the nutritional components that are critical for life-span extension will help to reveal the mechanisms by which it operates.

NUTRIENT intake has profound effects on development, fertility, and longevity. The ingested quantity of a nutritionally adequate diet is thought to dictate a trade-off between the ability to sustain vigorous growth or high fertility on the one hand, and the development of age-related pathologies that determine length of life on the other (1). Thus, it appears that the factors that contribute to the reproductive success of an organism in the face of competition are the very things that contribute to its decline with age. The theoretical work (1–4) and empirical studies (5–9) that have examined this trade-off provide an evolutionary framework for the study of the relationship between nutrition and life span.

Dietary restriction (DR) appears to be a truly ''public'' modulator of life span (10) because it has been shown to increase longevity in organisms ranging from yeast (11,12), various invertebrates (6,13), and rodents (14) to primates, where preliminary data indicate that it is having positive results (15–18). Although conservation of the longevity phenotype spans the evolutionary distance from single-celled organisms to mammals, little is known of the mechanisms that connect diet and life span in the different species. For progress to be made in this area, we believe it is important that two criteria are fulfilled in studies of DR: 1) protocols are demonstrated to operate within the evolutionary trade-offs described above so that at the food-intake level for increased longevity (restricted), fecundity and/or growth are coordinately reduced, demonstrating that the relatively short-lived animals (fully fed) are likely to have increased evolutionary fitness; and 2) the diets are well defined. These rules allow dietary manipulations to be related to specific physiological responses that should inform us about the mechanisms by which diet affects the risk of death.

A great deal of literature has been published concerning the effects of DR on rodent life span since the first report in 1935 by McCay and colleagues (14), and many good comprehensive reviews are available on this topic (19–22). However, 70 years of research on DR has yielded relatively few insights into the mechanisms by which this intervention works to extend life. One experimental approach to elucidating these mechanisms is the study of food components that are critical for the effects of DR. We therefore begin this review by considering the potential role that calories have been proposed to play in longevity. To address the role of many more dietary components on life span in a thorough manner, it is appropriate to use relatively short-lived and easily contained model organisms. Data from such model-organism studies can then be used to provide direction for the more laborious and expensive work of testing dietary manipulations on mammals. It is our aim, therefore, to highlight the research value of the model organism *Drosophila melanogaster* for detailed DR studies and to provide guidelines for their appropriate experimental design. Finally, we also provide a brief summary of several important mechanistic insights into DR that have already been made using *D. melanogaster* that currently await testing in mammalian systems.

Background

Features of Food-Reduction-Induced Extension of Life Span

DR has been established as a robust and repeatable method for extending life span in rodents. A broad range of nutritional restriction from 33% to 80% of ad libitum intake has been used to extend both mean and maximum life span of rodents (19). It is also of great interest that the longevity-promoting qualities of reduced food intake have been shown to be effective when the regimen is administered postdevelopmentally, indicating that the protective effects of food reduction can be acquired later in life and do not necessarily come at the cost of delayed development (7,23–25).

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Why would protein be so important in regulation aging?

Life is a constant battle between damage and repair

Excess Protein increases damage
and reduces ability to repair it

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Reduction of food intake without malnourishment extends life span in many different organisms. The majority of work in this field has been performed in rodents where it has been shown that both restricting access to the entire diet and restricting individual dietary components can cause life-span extension. Thus, for insights into the mode of action of this intervention, it is of great interest to investigate the aspects of diet that are critical for life span extension. Further studies on the mechanisms of how food components modify life span are well suited to the model organism *Drosophila melanogaster* because of its short life span and ease of handling and containment. Therefore, we summarize practical aspects of implementing dietary restriction in this organism, as well as highlight the major advances already made. Delineation of the nutritional components that are critical for life-span extension will help to reveal the mechanisms by which it operates.

NUTRIENT intake has profound effects on development, fertility, and longevity. The ingested quantity of a nutritionally adequate diet is thought to dictate a trade-off between the ability to sustain vigorous growth or high fertility on the one hand, and the development of age-related pathologies that determine length of life on the other (1). Thus, it appears that the factors that contribute to the reproductive success of an organism in the face of competition are the very things that contribute to its decline with age. The theoretical work (1–4) and empirical studies (5–9) that have examined this trade-off provide an evolutionary framework for the study of the relationship between nutrition and life span.

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Protein is the most thermogenic macronutrient

Protein...

increases glycation

oxidative damage

Increases IGF-1

Kidney Int. 2005 Mar;67(3):953-68.

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Amino acids injure mesangial cells by advanced glycation end products, oxidative stress, and protein kinase C.

Tuttle KR,Johnson EC, Cooney SK, Meek RL

The Heart Institute of Spokane and Sacred Heart Medical Center, Spokane, Washington 99204, USA.

designed to resemble protein feeding, high glucose (30.5 mmol/L), and, the combination, amino acids/high glucose. AGEs, reactive oxygen species (ROS), protein kinase C (PKC) activity and production, and mitogen-activated protein (MAP) kinase-extracellular signal regulated kinase (ERK) 1,2 activity were measured. Inhibitors were used to determine roles of these processes in fibrosis and/or AGE formation. RESULTS: AGE immunostaining increased when cells were cultured in amino acids and was comparable to that observed with high glucose. In amino acids/high glucose, AGE immunostaining appeared even greater. Amino acids, high glucose, and amino acids/high glucose induced ROS production. Aminoguanidine and vitamin E prevented AGE accumulation and induction of protein and mRNA for fibrosis markers [transforming growth factor-beta1 (TGF-beta1), fibronectin, and collagen IV]. PKC and ERK 1,2 activity increased with amino acids, high glucose, and amino acids/high glucose. PKC-beta inhibition prevented ERK 1,2 activation and fibrosis induction. ERK 1,2 inhibition also blocked the fibrosis response. CONCLUSION: A profibrotic injury response occurred in mesangial cells exposed to amino acids, with or without high glucose, by formation of AGE, oxidative stress, and activation of the PKC-beta and MAP kinase-ERK 1,2 signal pathway. These observations provide new insight into cellular mechanisms of kidney damage produced by excess dietary protein, particularly in diabetes.

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Caloric Restriction, Slowing Aging, and Extending Life

Edward J. Masoro

(Published 26 February 2003)
REVIEW
sageke.sciencemag.org/cgi/content/full/sageke;2003/8/re2

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Introduction

In an article entitled "The History of Gerontology," Jim Birren

duced food intake on aging and longevity in rats (*4*, *5*). They blunted the growth of one group of female rats by decreasing their food intake over the period from 45 days of age to 6

stricting the caloric intake of several poikilothermic and homeothermic species have been found to increase the maximal length of life of these species (*2*). There are claims that other environmental manipulations and the administration of a variety of chemical agents increase the maximal length of life, but none of these exhibit a consistently observed robust effect. Indeed, only caloric restriction has resulted in a consistent robust increase in the maximal length of life in mammalian species, specifically rats and mice.

sue further, a decision that resulted in their classic research on caloric restriction.

Before 1930

In 1914, Francis Peyton Rous (who was to receive a Nobel Prize in 1966 for his work on cancer) published a paper in the *Journal of Experimental Medicine* that showed that reducing food intake inhibited the occurrence of spontaneous tumors in rodents (*3*). Although this paper did not directly address the effects of caloric restriction on longevity and aging, it was the first in a long line of reports showing that decreasing food intake retards carcinogenesis. Moreover, because the onset of most cancers is age-associated, many of those reports have ad-

1930s Studies of McCay and Colleagues

In their first study, begun in 1930, they used three groups of weanling rats (*8*). One group, fed ad libitum, grew to maturity at what the investigators felt to be a normal rate. Food intake was restricted for the other two groups, so that no growth occurred until death seemed imminent, whereupon the allotment of food was increased just enough to keep the rats alive. Thus, the rats on the restricted food intake underwent long periods of no growth interspersed with periods of growth. One of the two groups was kept on the restricted diet for 700 days and the other for 900 days. The rats that grew normally had a mean length of life of about 600 days, but many of the rats in the two restricted groups lived much longer than that.

The first study of McCay and associates had involved the restriction of all components of the diet. In a subsequent study (*9*), the intake of fat and carbohydrate was restricted but not that of protein, minerals, and vitamins. Again, the group of rats on the restricted rations lived much longer.

McCay and colleagues concluded that the longer length of life of rats on the restricted diets was due to the decreased rate of growth. However, it should be noted that such a conclusion

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Reducing Protein Extends Life

Aging Cell. 2006 Aug;5(4):305-14. Epub 2006 Jun 26.

Methionine restriction decreases visceral fat mass and preserves insulin action in aging male Fischer 344 rats independent of energy restriction.

• Malloy VL, Krajcik RA, Bailey SJ, Hristopoulos G, Plummer JD, Orentreich N.

Reduced dietary methionine intake (0.17% methionine, MR) and calorie restriction (CR) prolong lifespan in male Fischer 344 rats. Although the mechanisms are unclear, both regimens feature lower body weight and reductions in adiposity. Reduced fat deposition in CR is linked to preservation of insulin responsiveness in older animals. These studies examine the relationship between insulin responsiveness and visceral fat in MR and test whether, despite lower food intake observed in MR animals, decreased visceral fat accretion and preservation of insulin sensitivity is not secondary to CR. Accordingly, rats pair fed (pf) control diet (0.86% methinone, CF) to match the food intake of MR for 80 weeks exhibit insulin, glucose, and leptin levels similar to control-fed animals and comparable amounts of visceral fat. Conversely, MR rats show significantly reduced visceral fat compared to CF and PF with concomitant decreases in basal insulin, glucose, and leptin, and increased adiponectin and triiodothyronine. Daily energy expenditure in MR animals significantly exceeds that of both PF and CF. In a separate cohort, insulin responses of older MR animals as measured by oral glucose challenge are similar to young animals. Longitudinal assessments of MR and CF through 112 weeks of age reveal that MR prevents age-associated increases in serum lipids. By 16 weeks, MR animals show a 40% reduction in insulin-like growth factor-1 (IGF-1) that is sustained throughout life; CF IGF-1 levels decline much later, beginning at 112 weeks. Collectively, the results indicate that MR reduces visceral fat and preserves insulin activity in aging rats independent of energy restriction.
PMID: 16800846 [PubMed - in process]

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Nutritional Control of Aging

Jay A. Zimmerman[1,2], Virginia Malloy[1], Rozlyn Krajcik[1], Norman Orentreich[1]
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For more than 60 years the only dietary manipulation known to retard aging was caloric restriction, in which a variety of species respond to a reduction in energy intake by demonstrating extended median and maximum life span. More recently, two alternative dietary manipulations have been reported to also extend survival in rodents. Reducing the tryptophan content of the diet extends maximum life span, while lowering the content of sulfhydryl-containing amino acids in the diet by removing cysteine and restricting the concentration of methionine has been shown to extend all parameters of survival, and to maintain blood levels of the important anti-oxidant glutathione. To control for the possible reduction in energy intake in methionine-restricted rats, animals were offered the control diet in the quantity consumed by rats fed the low methionine diet. Such pair-fed animals experienced life span extension, indicating that methionine restriction-related life span extension is not a consequence of reduced energy intake. By feeding the methionine restricted diet to a variety of rat strains we determined that lowered methionine in the diet prolonged life in strains that have differing pathological profiles in aging, indicating that this intervention acts by altering the rate of aging, not by correcting some single defect in a single strain.

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Journal of Gerontology: BIOLOGICAL SCIENCES
2005, Vol. 60A, No. 5, 549–555

Counting the Calories: The Role of Specific Nutrients in Extension of Life Span by Food Restriction

Matthew D. W. Piper, William Mair, and Linda Partridge

Department of Biology, University College London, United Kingdom.

Reduction of food intake without malnourishment extends life span in many different organisms. The majority of work in this field has been performed in rodents where it has been shown that both restricting access to the entire diet and restricting individual dietary components can cause life-span extension. Thus, for insights into the mode of action of this intervention, it is of great interest to investigate the aspects of diet that are critical for life span extension. Further studies on the mechanisms of how food components modify life span are well suited to the model organism *Drosophila melanogaster* because of its short life span and ease of handling and containment. Therefore, we summarize practical aspects of implementing dietary restriction in this organism, as well as highlight the major advances already made. Delineation of the nutritional components that are critical for life-span extension will help to reveal the mechanisms by which it operates.

NUTRIENT intake has profound effects on development, fertility, and longevity. The ingested quantity of a nutritionally adequate diet is thought to dictate a trade-off between the ability to sustain vigorous growth or high fertility on the one hand, and the development of age-related pathologies that determine length of life on the other (1). Thus, it appears that the factors that contribute to the reproductive success of an organism in the face of competition are the very things that contribute to its decline with age. The theoretical work (1–4) and empirical studies (5–9) that have examined this trade-off provide an evolutionary framework for the study of the relationship between nutrition and life span.

Dietary restriction (DR) appears to be a truly ''public'' modulator of life span (10) because it has been shown to increase longevity in organisms ranging from yeast (11,12), various invertebrates (6,13), and rodents (14) to primates, where preliminary data indicate that it is having positive results (15–18). Although conservation of the longevity phenotype spans the evolutionary distance from single-celled organisms to mammals, little is known of the mechanisms that connect diet and life span in the different species. For progress to be made in this area, we believe it is important that two criteria are fulfilled in studies of DR: 1) protocols are demonstrated to operate within the evolutionary trade-offs described above so that at the food-intake level for increased longevity (restricted), fecundity and/or growth are coordinately reduced, demonstrating that the relatively short-lived animals (fully fed) are likely to have increased evolutionary fitness; and 2) the diets are well defined. These rules allow dietary manipulations to be related to specific physiological responses that should inform us about the mechanisms by which diet affects the risk of death.

A great deal of literature has been published concerning the effects of DR on rodent life span since the first report in 1935 by McCay and colleagues (14), and many good comprehensive reviews are available on this topic (19–22). However, 70 years of research on DR has yielded relatively few insights into the mechanisms by which this intervention works to extend life. One experimental approach to elucidating these mechanisms is the study of food components that are critical for the effects of DR. We therefore begin this review by considering the potential role that calories have been proposed to play in longevity. To address the role of many more dietary components on life span in a thorough manner, it is appropriate to use relatively short-lived and easily contained model organisms. Data from such model-organism studies can then be used to provide direction for the more laborious and expensive work of testing dietary manipulations on mammals. It is our aim, therefore, to highlight the research value of the model organism *Drosophila melanogaster* for detailed DR studies and to provide guidelines for their appropriate experimental design. Finally, we also provide a brief summary of several important mechanistic insights into DR that have already been made using *D. melanogaster* that currently await testing in mammalian systems.

Background

Features of Food-Reduction-Induced Extension of Life Span

DR has been established as a robust and repeatable method for extending life span in rodents. A broad range of nutritional restriction from 33% to 80% of ad libitum intake has been used to extend both mean and maximum life span of rodents (19). It is also of great interest that the longevity-promoting qualities of reduced food intake have been shown to be effective when the regimen is administered postdevelopmentally, indicating that the protective effects of food reduction can be acquired later in life and do not necessarily come at the cost of delayed development (7,23–25).

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Counting the Calories: The Role of Specific Nutrients in Extension of Life Span by Food Restriction

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Recent work has shown that DR in D. melanogaster is the product of an acute effect (on genetic transcription) that causes fully-fed flies to adopt the mortality profile of lifelong DR flies within 48 hours of initiation of the treatment; this finding indicates that DR has no effect on the accumulation of irreversible, ageing-related damage ...It will be of great interest to see if this acute reversal is conserved in mammals, as has been suggested by the reversal of transcript profiles after short-term dietary changes.

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Alterations in nutrition-related signalling pathways are thought to initiate the cascade of changes that underlie longevity assurance by dietary alterations.

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...insulin and insulin-like growth factor (IGF) signalling can alter life span in rodents (70,71), D. melanogaster (72–75), and C. elegans (76–78). Insulin signalling is known to be involved in the regulation of energy homeostasis in response to diet, thus providing an attractive link between the caloric contents of diets and their action in extending life span.

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However, another signalling pathway has recently expanded the field, because modifications to TOR signalling, also involved in metabolic homeostasis (*principally in response to **protein***), can alter life span (81–84). These studies have provided further support for the argument that the protective effects of dietary reduction are not limited to calories alone, but involve an aspect of ***protein*** metabolism as well.

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A little more on mTOR

The amino acid sensitive TOR pathway from yeast to mammals

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Abstract **The target of rapamycin (TOR) is an ancient effector of cell growth that integrates signals from growth factors and nutrients. Two downstream effectors of mammalian TOR, the translational components S6K1 and 4EBP1, are commonly used as reporters of mTOR activity. The conical signaling cascade initiated by growth factors is mediated by PI3K, PKB, TSC1/2 and Rheb. However, the process through which nutrients, i.e., amino acids, activate mTOR remains largely unknown. Evidence exists for both an intracellular and/or a membrane bound sensor for amino acid mediated mTOR activation. Research in eukaryotic models, has implicated amino acid transporters as nutrient sensors. This review describes recent advances in nutrient signaling that impinge on mTOR and its targets including hVps34, class III PI3K, a transducer of nutrient availability to mTOR.**

Keywords: mTOR; Amino acid transport; Leucine; hVPS34

1. Introduction: Insulin stimulation and mTOR signaling

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Stimulation of PI3K by growth factors such as insulin results in the initiation of a number of signaling cascades that lead to growth and proliferation, a cellular phenomenon conserved throughout metazoans (Fig. 1). In mammals, receptor interaction with insulin results in recruitment of insulin receptor substrates (IRS) to the cell membrane [4]. Subsequently, recruitment and stimulation of the class I PI3K produces the phosphatidylinositol second messenger PIP_3 [5]. PIP_3 binds to the pleckstrin homology (PH) domain of several proteins, in particular to the Protein A, G and C (AGC) serine/threonine kinase PKB/AKT, a pro-growth, pro-survival kinase [6]. Binding of PIP_3 to its PH domain recruits PKB to the cell membrane where it is activated through phosphorylation by PDK1 and PDK2 [7,8]. Activated PKB phosphorylates the TSC1/2 complex resulting in their dissociation and degradation, thereby releasing the small GTPase Rheb from the inhibitory GAP activity of TSC2 [9–13]. Recent studies illustrate a direct interaction between Rheb and mTOR, which stimulates its kinase activity [14].

Two alleles, TOR1p and TOR2p, were originally described in a screen of yeast mutants resistant to toxic doses of the anti-fungal, bacterial macrolide, rapamycin [15]. In mammals, rapamycin blocks mTOR function by forming an inhibitory complex with the immunophilin FKBP12, which binds to and attenuates the ability of mTOR to phosphorylate downstream substrates [16,17], including S6K1 [3,18–20] as well as the 4E binding protein, 4E-BP1 [21–23], a repressor of translation initiation factor 4E [24]. In this rapamycin sensitive pathway mTOR is bound to two additional proteins, raptor and mLst8/GβL, to make Complex 1. Raptor and mLst8 are homologues of the yeast KOG1p and Lst8p, respectively [25–27]. mTOR interacts with downstream substrates through raptor, which recognizes mTOR substrates through their TOR signaling (TOS) motifs [28], whereas mLST8 is required to make a competent signaling complex that can respond to nutrient and energy inputs [27]. Recent studies show that mTOR also exists in a second signaling complex with mLST8 and a protein termed rictor, rather than raptor. Moreover, this second complex is proposed to directly control PKB phosphorylation and activation and, unlike Complex 1, Complex 2 is rapamycin resistant [8,29].

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The amino acid sensitive TOR pathway from yeast to mammals

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Rheb. However, the process through which nutrients, i.e., amino acids, activate mTOR remains largely unknown. Evidence exists for both an intracellular and/or a membrane bound sensor for amino acid mediated mTOR activation. Research in eukaryotic models, has implicated amino acid transporters as nutrient sensors. This review describes recent advances in nutrient signaling that impinge on mTOR and its targets including hVps34, class III PI3K, a transducer of nutrient availability to mTOR.

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Future studies that examine the link between amino acid transport, hVPS34 activity and mTOR activity will resolve the initial signaling events that result in activation of mTOR targets and provide insight into the opportunistic scavenging of nutrients by malignant cells.

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Inhibition of Mammalian Target of Rapamycin Activates Apoptosis Signal-regulating Kinase 1 Signaling by Suppressing Protein Phosphatase 5 Activity*

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rapamycin caused rapid dissociation of the PP2A-B″ regulatory subunit (PR72) from the PP5-ASK1 complex, which was associated with reduced phosphatase activity of PP5. This effect was dependent on expression of eukaryotic initiation factor 4E-binding protein 1 (4E-BP1). Down-regulation of PP5 activity by rapamycin coordinately activated ASK1, leading to elevated phosphorylation of c-Jun. Amino acid deprivation, which like rapamycin inhibits mTOR signaling, also inhibited PP5 activity, caused rapid dissociation of PR72, and activated ASK1 signaling. Overexpression of PP5, but not the PP2A catalytic subunit, blocked rapamycin-induced phosphorylation of c-Jun, and protected cells from rapamycin-induced apoptosis. The results suggest that PP5 is downstream of mTOR, and positively regulated by the mTOR pathway. The findings suggest that in the absence of serum factors, mTOR signaling suppresses apoptosis through positive regulation of PP5 activity and suppression of cellular stress.

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binding protein 12 (FKBP-12) and this complex binds mTOR and inhibits its function. Subsequently, eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) becomes hypophosphorylated and associates with eIF4E (15, 16). This association prevents binding of eIF4E to eIF4G and formation of eIF4F initiation complex, thereby inhibiting cap-dependent translation of mRNA. Inhibition of mTOR by rapamycin also directly or indirectly inactivates ribosomal p70S6 kinase (S6K1), thereby blocking translation of mRNA species containing 5′-terminal oligopyrimidine tracts (TOP) (17, 18). However, the requirement for S6K1 activity in translation of TOP containing mRNAs has been recently challenged. Importantly, complete inhibition of mTOR by rapamycin had only a slight repressive effect on translation of TOP mRNAs leading to the conclusion that regulation by growth factors and mitogens is primarily through the PI3K pathway with a minor role for mTOR in regulation of TOP mRNA translation (19, 20). In many cell lines, exposure to rapamycin reduces overall protein synthesis only ~15–20% but results in a specific G_1 accumulation.

We previously found that proliferation of human rhabdomyosarcoma cells was inhibited by low concentrations of rapamycin (21, 22). Under serum-free culture conditions rapamycin treatment induces apoptosis in these and other cells lacking functional p53 (12, 13). Ectopic expression of p53 or $p21^{Cip1}$ protects cells from apoptosis (13). Rapamycin inhibition of mTOR induces a cellular stress response characterized by rapid and sustained activation of apoptosis signal-regulating kinase 1 (ASK1) signaling in p53-mutant cells. In contrast only transient activation of ASK1 signaling occurs in cells express-

[1] The abbreviations used are: mTOR, mammalian target of rapamycin; ASK1, apoptosis signal-regulating kinase 1; PP2A, protein phosphatase 2A; PP5, protein phosphatase 5; PR72, PP2A-B″ regulatory subunit; 4E-BP1, eukaryotic initiation factor 4E-binding protein 1; S6K1, p70S6 kinase; TPR, tetratricopeptide repeat; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; PBS, phosphate-buffered saline; FACS, fluorescent-activated cell signaling; TOP, 5′-terminal oligopyrimidine tracts; FITC, fluorescein isothiocyanate.

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The Tor Pathway Regulates Gene Expression by Linking Nutrient Sensing to Histone Acetylation

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nutrient limitation. Our results illustrate that the Tor pathway links nutrient sensing with histone acetylation to control RP gene expression and cell growth.

The rapamycin-sensitive Tor signaling pathway couples nutrient availability with cell growth in *Saccharomyces cerevisiae*, *Drosophila melanogaster*, and mammalian cells (36, 38, 41). In addition to a well-established role in the control of translation initiation, the Tor proteins play a dynamic role in controlling transcription in response to nutrient signals. Inhibition of the Tor kinases by rapamycin or nitrogen limitation results in the marked induction of genes subject to nitrogen catabolite repression (NCR), genes associated with the retrograde response, and genes induced by various environmental stresses such as sodium toxicity and carbon starvation (2, 4, 6, 9, 13, 19). In contrast, inhibition of Tor results in the rapid repression of genes involved in ribosome biogenesis, including tRNAs and rRNAs transcribed by Pol I and Pol III as well as ribosomal proteins expressed by Pol II (6, 25, 35, 49). Significant progress has been made in understanding how the genes that are induced by Tor inhibition are controlled. In these cases, under optimal nutrient conditions the Tor pathway prevents the nuclear import of the corresponding transcription factors, including Gln3, Gat1, Msn2, Msn4, Rtg1, and Rtg3 (2, 4, 19). However, the molecular mechanism(s) by which Tor regulates expression of the RP genes remains poorly understood.

The RP genes are subject to stringent regulation in order to couple protein synthesis and growth to the availability of nutrients and the physiological status of the cell (48). In addition to the Tor pathway, two other important signaling pathways regulate RP gene expression. The nutrient-sensing protein kinase A (PKA) pathway is required to activate RP gene expression while the PKC pathway mediates repression of RP genes in response to perturbations of the cell integrity pathway (18, 31). Additional signaling programs are also thought to regulate RP gene expression in response to nutrients (30). The majority of RP gene promoters contain binding sites for two transcription factors of partially overlapping function: Abf1 and Rap1 (21, 23; reviewed in reference 33). The most prominent of these factors, Rap1, activates RP gene expression and also functions in silencing telomeres and the silent mating type loci *HML* and *HMR* (15, 47).

Gene activation in eukaryotic cells requires mechanisms that overcome the repressive effects of chromatin at specific promoters. A growing body of evidence suggests that this is accomplished by the recruitment of chromatin-remodeling complexes by site-specific transactivators (46). Recent work has demonstrated a strong correlation between recruitment of the Esa1 histone acetylase and transcription from RP gene promoters (37). Furthermore, recruitment of Esa1 to RP gene promoters requires a binding site for Rap1 and/or Abf1 (37). Esa1 is the catalytic subunit of the NuA4 histone acetylase complex that acetylates histones H4 and H2A (1). The NuA4 complex is recruited to DNA by acidic activators such as VP16 and Gcn4 (5).

In this work we examined whether Tor signaling is required for the occupancy of known regulatory factors at the RP gene promoters by using chromatin immunoprecipitation assays. We found that Tor signaling is required for the maintenance of Esa1 at RP gene promoters. Repression of RP genes in response to nutrient depletion or rapamycin treatment requires components of the Rpd3-Sin3 histone deacetylase complex. Our results establish a link between Tor-mediated nutritional signaling and histone acetylation and illustrate a novel mechanistic paradigm by which the Tor pathway controls gene expression.

MATERIALS AND METHODS

Saccharomyces cerevisiae* strains, plasmids, and growth conditions.** Strain MCY47 was obtained by introducing a three-hemagglutinin (HA) epitope-tagged Esa1 in a two-step gene replacement (with plasmid YIplac211 HA-Esa1, a generous gift from Kevin Struhl) into strain MLY41 Σ1278b *MATa** *ura3-52* (37). Strains JRY16a, JRY17a, and JRY18a were derived from MLY41a by replacing the entire open reading frame of *RPD3*, *SIN3*, and *SAP30*, respectively, with *kanMX*. Gene disruptions were all verified by PCR.

Chromatin immunoprecipitation and quantitative PCR. Exponentially growing cultures of strain MCY47 containing HA_3 epitope-tagged Esa1 were treated with 100 nM rapamycin for 0, 15, 30, and 60 min. Cultures were adjusted to 1% formaldehyde and incubated for 20 min at room temperature with gentle shak-

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John R. Rohde and Maria E. Cardenas*

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...rapamycin is now being developed to prevent restenosis following cardiac stent surgery and most recently as a novel chemotherapy agent (16, 26, 43). The promise of rapamycin as a cancer drug is being explored in phase II and III clinical trials and recent reports have demonstrated its remarkable antitumor activity in cells.

has been made in understanding how the genes that are induced by Tor inhibition are controlled. In these cases, under optimal nutrient conditions the Tor pathway prevents the nuclear import of the corresponding transcription factors, including Gln3, Gat1, Msn2, Msn4, Rtg1, and Rtg3 (2, 4, 19). However, the molecular mechanism(s) by which Tor regulates expression of the RP genes remains poorly understood.

The RP genes are subject to stringent regulation in order to couple protein synthesis and growth to the availability of nutrients and the physiological status of the cell (48). In addition to the Tor pathway, two other important signaling pathways regulate RP gene expression. The nutrient-sensing protein kinase A (PKA) pathway is required to activate RP gene expression while the PKC pathway mediates repression of RP genes in response to perturbations of the cell integrity pathway (18, 31). Additional signaling programs are also thought to regulate RP gene expression in response to nutrients (30). The majority of RP gene promoters contain binding sites for two transcription factors of partially overlapping function: Abf1 and Rap1

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complex is recruited to DNA by acidic activators such as VP16 and Gcn4 (5).

In this work we examined whether Tor signaling is required for the occupancy of known regulatory factors at the RP gene promoters by using chromatin immunoprecipitation assays. We found that Tor signaling is required for the maintenance of Esa1 at RP gene promoters. Repression of RP genes in response to nutrient depletion or rapamycin treatment requires components of the Rpd3-Sin3 histone deacetylase complex. Our results establish a link between Tor-mediated nutritional signaling and histone acetylation and illustrate a novel mechanistic paradigm by which the Tor pathway controls gene expression.

MATERIALS AND METHODS

Saccharomyces cerevisiae* strains, plasmids, and growth conditions.** Strain MCY47 was obtained by introducing a three-hemagglutinin (HA) epitope-tagged Esa1 in a two-step gene replacement (with plasmid YIplac211 HA-Esa1, a generous gift from Kevin Struhl) into strain MLY41 Σ1278b *MATa** *ura3-52* (37). Strains JRY16a, JRY17a, and JRY18a were derived from MLY41a by replacing the entire open reading frame of *RPD3*, *SIN3*, and *SAP30*, respectively, with *kanMX*. Gene disruptions were all verified by PCR.

Chromatin immunoprecipitation and quantitative PCR. Exponentially growing cultures of strain MCY47 containing HA_3 epitope-tagged Esa1 were treated with 100 nM rapamycin for 0, 15, 30, and 60 min. Cultures were adjusted to 1% formaldehyde and incubated for 20 min at room temperature with gentle shak-

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We therefore find it intriguing that, in mammalian cells, rapamycin treatment results in a gene expression profile that resembles one seen with amino acid limitation (32).

in response to perturbations of the cell integrity pathway (18, 31). Additional signaling programs are also thought to regulate RP gene expression in response to nutrients (30). The majority of RP gene promoters contain binding sites for two transcription factors of partially overlapping function: Abf1 and Rap1

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Extension of chronological life span in yeast by decreased TOR pathway signaling

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Decreased TOR activity also promoted increased accumulation of storage carbohydrates and enhanced stress resistance and nuclear relocalization of the stress-related transcription factor Msn2. We propose that up-regulation of a highly conserved response to starvation-induced stress is important for life span extension by decreased TOR signaling in yeast and higher eukaryotes.

[*Keywords*: *Saccharomyces cerevisiae*; TOR; aging; life span; nutrients; yeast]

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Although aging and age-related diseases account for enormous social and economic costs, the mechanisms that underlie the gradual and progressive deterioration observed in humans remain poorly understood. However, research on model organisms such as mice, flies, worms, and yeast has provided insights into both genetic and environmental factors that can control aging. As with humans, each of these model organisms displays an exponential increase in mortality as it ages (Kaeberlein et al. 2001). Model organisms are useful for their similar basic biology and relatively short life span. Furthermore, the advent of reagents such as the genome-wide deletion collection of *Saccharomyces cerevisiae* strains (Winzeler et al. 1999) and high complexity *Caenorhabditis elegans* RNA interference (RNAi) libraries (Kamath et al. 2003; Rual et al. 2004) has made it feasible to conduct genome-wide screens for phenotypes, such as life span, that are difficult to screen by traditional methods. These tools have helped reveal genetic programs associated with increased longevity (Kenyon 1996, 2005; Kaeberlein 2004).

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A growing body of data from studies on model systems indicates that aspects of aging have been conserved throughout evolution, because similar interventions can increase life span among evolutionarily divergent species. One such intervention is calorie restriction (CR), which can slow aging in virtually every biological system examined (Weindruch and Walford 1988; Masoro 2005). In addition to increasing life span, CR induces many similar physiological changes in diverse species (Longo and Finch 2003). For example, increased stress resistance, decreased ribosome biogenesis, and metabolic reprogramming in response to nutrient depletion are hallmarks of CR in yeast, worms, flies, and mammals.

Reduced activity of nutrient-responsive insulin-like signaling pathways is also associated with enhanced longevity in multicellular eukaryotes. For example, mutation of the *C. elegans* insulin/IGF-1 receptor homolog, *Daf-2*, dramatically increases life span and up-regulates stress response genes through the FOXO-like transcription factor *Daf-16* (Kenyon et al. 1993; Kimura et al. 1997; Lin et al. 1997; Ogg et al. 1997). A similar pathway appears to regulate longevity in flies, as well (Hwangbo et al. 2004), and subsequent work in mice demonstrated that a variety of mutations conferring endocrine deficits

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TOR signaling regulates multiple cellular processes in response to nutrients, especially amino acids, raising the possibility that decreased TOR signaling mediates life span extension by calorie restriction. In support of this possibility, removal of either asparagine or glutamate from the media significantly increased stationary phase survival. Pharmacological inhibition of TOR signaling by methionine sulfoximine or rapamycin also increased CLS.

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Distinct Signaling Events Downstream of mTOR Cooperate To Mediate the Effects of Amino Acids and Insulin on Initiation Factor 4E-Binding Proteins

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no such preference. These data have important implications for understanding signaling downstream of mTOR and the development of new strategies to impair mTOR signaling.

There is presently a high level of research interest in signaling through the mammalian target of rapamycin (mTOR). This reflects its key roles in regulating cell and animal growth, the cell cycle, and gene expression (transcription and translation) (16, 28, 60). Recent studies have demonstrated mTOR is essential for cell growth and proliferation (53). Furthermore, rapamycin, a specific inhibitor of mTOR signaling, is in clinical use, or has clinical potential, for graft rejection (45), restenosis after angioplasty (13), certain types of cancer (33), tumor angiogenesis (25, 77), and liver fibrosis (78). Important recent advances have identified new components on this pathway involved in the upstream control of mTOR signaling (e.g., TSC1, TSC2, and the small G-protein Rheb) (20, 40, 47, 50) and in downstream signaling from mTOR, which involves complexes with partner proteins (e.g., raptor [26, 37], GβL [36], and their yeast orthologs [44]). Nonetheless, our overall understanding of the pathway remains far from complete.

The best understood targets of the mTOR pathway are proteins that regulate the translational machinery. One intensively studied target is the translational repressor protein, 4E-BP1 (eukaryotic initiation factor 4E-binding protein 1) (23, 41). Binding of 4E-BP1 to eIF4E prevents the latter protein from engaging with other partners, such as the scaffold eIF4G, and therefore blocks cap-dependent mRNA translation initiation. 4E-BP1 undergoes phosphorylation at multiple sites (Fig. 1A), and phosphorylation at some of them disrupts its ability to bind eIF4E, leading to release of 4E-BP1 and allowing eIF4E to bind eIF4G. Release of 4E-BP1 from eIF4E is generally blocked by rapamycin, indicating an essential role for mTOR signaling.

Phosphorylation of 4E-BP1 at several sites is stimulated by agents such as insulin, and, in many cell types, this effect requires the presence in the cells' medium of amino acids (60). Phosphorylation of 4E-BP1 is hierarchical, with phosphorylation of Thr36/45 being required for modification of Thr69 (the numbering of residues in human 4E-BP1 is shifted by +1 relative to the rodent orthologs; this site is therefore Thr70 in human 4E-BP1 [PHAS-I]) and Ser64 (21–23, 32, 51, 52). Phosphorylation of other sites, such as Ser101, appears not to be regulated (73).

Which kinase(s) is responsible for the complex phosphorylation of 4E-BP1 in vivo? mTOR can phosphorylate 4E-BP1 in vitro, at least under specific conditions (5, 7, 21, 49, 74). The fact that the C-terminal TOR-signaling (TOS) motif in 4E-BP1 (62) recruits raptor, and thus also mTOR, to 4E-BP1 and plays an important role in the phosphorylation of Ser64/5 and Thr69/70 in vivo appears consistent with the idea that mTOR might indeed directly phosphorylate several sites in 4E-BP1 within cells (3, 9, 55, 63). Rapamycin may disrupt mTOR/raptor complexes (37, 56), which could help explain how rapamycin interferes with the phosphorylation of 4E-BP1. This has given rise to the widely accepted notion that the kinase activity of mTOR directly phosphorylates 4E-BP1 and that inhibition of 4E-BP1 phosphorylation by rapamycin reflects impairment of this activity. However, in vitro, mTOR primarily phosphor-

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Amino acid signalling and the integration of metabolism☆

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Abstract

It has become clear in recent years that amino acids are not only important as substrates for various metabolic pathways but that they can also activate a nutrient-sensitive, mTOR-mediated, signalling pathway in synergy with insulin. Leucine is the most effective amino acid in this regard. The signalling pathway is antagonised by AMP-activated protein kinase. Amino acid signalling stimulates protein synthesis and inhibits (autophagic) proteolysis. In addition, many amino acids cause an increase in cell volume. Cell swelling per se stimulates synthesis of protein, glycogen, and lipid, in part by further stimulating signalling and in part by unrelated mechanisms. Amino acids also stimulate signalling in β-cells and stimulate β-cell growth and proliferation. This results in increased production of insulin, which enhances the anabolic (and anti-catabolic) properties of amino acids. Finally, amino acid-dependent signalling controls the production of leptin by adipocytes, and thus contributes to the regulation of appetite.

Keywords: Autophagy; p70S6 kinase; PI 3-kinase; mTOR; Tuberous sclerosis complex; Raptor; Rapamycin; Wortmannin; AMP-activated protein kinase; Insulin

The notion that amino acids are not only important as substrates for metabolic pathways but also function as regulators of metabolism is not new. For instance, glutamate controls urea synthesis via synthesis of *N*-acetylglutamate, the essential allosteric activator of carbamoyl-phosphate synthase [1]. Glutamate and aspartate kinetically control flux through the malate–aspartate shuttle, mediating the transfer of cytosolic reducing equivalents to the mitochondria, e.g., during aerobic glycolysis [2]. Leucine activates glutamate dehydrogenase, which contributes to the ability of leucine to potentiate insulin production in β-cells [3]. Some amino acids, leucine in particular, inhibit autophagy, which process, although quantitatively most important in the liver, occurs in almost all cell types [4,5]. Amino acid receptors in the brain are involved in neurotransmission [6]. Some amino acids induce cell swelling because of an increase in intracellular osmolarity following Na^+-dependent, concentrative, amino acid transport across the plasma membrane, and because of the intracellular accumulation of impermeant products (e.g., glutamate) in the course of their metabolism. Cell swelling per se mimics many of the effects of insulin in that it stimulates anabolic pathways (such as the synthesis of protein, glycogen, and lipid) and inhibits catabolism [7–9]. In the case of glycogen synthesis, part of the mechanism involved is direct activation of glycogen synthase phosphatase by a rise in intracellular glutamate and deinhibition of the enzyme by the fall in intracellular chloride which accompanies "regulatory volume decrease" [10].

New insight into the regulation of nitrogen metabolism by amino acids was obtained when we discovered that amino acids could stimulate a signalling pathway that is used by insulin. In search of a mechanism responsible for the inhibition of autophagy by amino acids in hepatocytes, we looked for proteins that might

☆ *Abbreviations:* IR, insulin receptor; IRS, insulin receptor substrate; PI3K, phosphatidylinositol 3-kinase; PI3P, phosphatidylinositol 3-phosphate; $PI45P_2$, phosphatidylinositol 4,5-bisphosphate; $PI345P_3$, phosphatidylinositol 3,4,5-trisphosphate; PDK1, phosphoinositide-dependent kinase 1; PKB, protein kinase B; TSC, tuberous sclerosis complex; mTOR, mammalian target of rapamycin; RVD, regulatory volume decrease; GS, glycogen synthase; ACC, acetylCoA carboxylase; AMPK, AMP-activated protein kinase; PP2A, protein phosphatase 2A; GAPP, glutamate-activated protein phosphatase.

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Amino acid signalling and the integration of metabolism[q]

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“amino acid infusion during an euglycaemic hyperinsulinemic clamp in fasted humans decreased rather than increased glucose disposal [60,61]. Although these data may be explained by substrate competition, i.e., the amino acids were oxidised instead of glucose, there are indications that amino acids, in fact, cause a time-dependent, rapamycin-sensitive, down-regulation of the activation of protein kinase B, and of glucose transport by insulin” [62

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proteasome-mediated proteolysis [55] and autophagic proteolysis [56] also decline with age, however. The latter two pathways can be considered as anti-aging repair mechanisms because they remove aberrant proteins and defective cell organelles. Interestingly, caloric restriction not only increases proteasome-mediated proteolysis [55] but also autophagic proteolysis which may contribute to increased longevity [56].

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The best drug to reduce mTor signalling, to slow aging and the chronic diseases associated with it, is already available...

Avoid high protein