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# Downregulation of plasma insulin levels and hepatic PPARγ expression during the first week of caloric restriction in mice

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**Abstract**

Calorie restriction extends lifespan by decreasing the rate of tumor formation, an effect occurring within 8 weeks of initiating a restricted diet. Our goal was to define how the first weeks of a calorie restricted diet (60% of ad libitum calories) affects putative mediators of the calorie restriction phenotype, focusing on regulators of fatty acid biosynthesis. In C57Bl/6 mice, insulin decreased over 50% ($p < 0.05$) during the first week of calorie restriction whereas IGF-1 was unaffected. In the liver, PPARγ mRNA fell to 13% of baseline after 1 week of calorie restriction ($p < 0.05$), whereas hepatic SREBP-1c and SIRT1 mRNA levels were unaffected. No changes in abdominal or subcutaneous adipose tissue were observed until after 4 weeks of caloric restriction. We conclude that calorie restriction-induced decreases in insulin and hepatic PPARγ are rapid enough to support a role for these molecules in triggering the initial phase of the calorie restriction phenotype.


*Keywords:* Aging; Caloric restriction; Lipogenesis; Mice; Liver; Adipose

## 1. Introduction

In all species studied to date, restricting caloric intake by 20–50% while still providing adequate micronutrients significantly extends mean and maximal lifespan, largely by retarding age-associated diseases, most importantly cancer (Weindruch, 1996; Weindruch et al., 1986). Not surprisingly there is intense interest in elucidating the molecular basis for the anti-aging and anti-cancer effects of calorie restriction (Ingram et al., 2006; Wolf, 2006). We have reported that 16 weeks of calorie restriction decreases protein levels in the liver of acetyl CoA carboxylase (ACC), the rate-limiting enzyme in fatty acid biosynthesis, to approximately 25% of baseline (Gonzalez et al., 2004). This observation is significant in that fatty acid biosynthesis appears to be closely linked to the timing and extent of tumor development, most notably in a subset of aggressive malignancies requiring a high rate of lipogenesis for growth (De Schrijver et al., 2003; Kuhajda, 2000; Menendez et al., 2005; Pizer et al., 1998). In fact, specific inhibition of fatty acid synthase (FAS) and lipogenesis have been used successfully to inhibit growth of malignancies (De Schrijver et al., 2003; Pizer et al., 1998). Thus calorie restriction-induced downregulation of ACC protein in the liver could be part of a general inhibition of fatty acid biosynthesis contributing to the observed decrease in spontaneous and induced tumor development in calorie restricted animals (Fu et al., 1994; Yoshida et al., 1999). Interestingly, the transcription factors PPARγ and SREBP-1c which regulate ACC and FAS expression have additional effects on processes central to aging such as cell cycling, apoptosis and inflammation (De Schrijver et al., 2003; Ettinger et al., 2004; Gonzalez et al., 2004; Kirkland et al., 2002; Picard and Guarente, 2005; Rossi et al., 2003; Semple et al., 2006). Thus any calorie restriction-induced

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downregulation of PPARγ, SREBP-1c, or other factors that influence fatty acid biosynthesis (i.e. insulin) could have collateral effects contributing to the anti-aging and anti-tumor phenotype of these animals.

Accordingly, our goal was to define how a calorie restricted diet affects the following regulators of fatty acid biosynthesis gene expression: PPARγ, SREBP-1c, SIRT1 and plasma insulin levels. Importantly, the caloric restriction phenotype of retarded aging and tumor development is evident within 8 weeks after initiating calorie restriction, with half of the eventual changes in hepatic gene expression already detectible at 2 weeks (Dhahbi et al., 2004). Thus the critical molecular events triggering this phenotype must occur within the first several weeks after initiating the restricted diet. Therefore, we focused on defining the early time course of caloric restriction's effects. To accomplish this, blood, liver and adipose tissue (subcutaneous and abdominal) were collected from 6-month-old C57Bl/6 mice that had undergone 0, 1, 4 or 16 weeks of calorie restriction (60% of ad libitum calories). We focused on liver because, while the main cause of neoplastic death of aging C57bl/6 mice is lymphoma not liver cancer, CR almost eliminates the incidence of liver tumors in this strain of mice whereas it has little effect on the rate of death due to lymphoma (Blackwell et al., 1995). For comparison to liver, another highly lipogenic tissue, white adipose tissue, was chosen, which itself has been suggested to play a key role in the calorie restriction phenotype (Wolf, 2006). Adipose tissue from two distinct anatomical sites was studied so that we could test the hypothesis that abdominal (epididymal) adipose tissue, which is more closely linked to pathology than subcutaneous adipose tissue, is resistant to calorie restriction-induced changes in gene expression (Lafontan and Berlan, 2003).

## 2. Methods

### 2.1. Animals

Mice ($N = 36$ C57Bl/6 males) were housed singly in the specific pathogen-free Shared Aging Rodent Facility at the Madison VA Geriatric Research, Education and Clinical Center, and provided a nonpurified diet (PLI 5001 [Purina Labs, St. Louis, MO]) and acidified water ad libitum for 3 weeks following weaning. From this time until 2 months of age, all 36 mice received 12 kcal/day of a semipurified diet (TD91349 [Teklad, Madison, WI]), which is ~10% less than the average ad libitum intake. The decision to impose a slight restriction on food intake during this period (and throughout life in control mice) was made because it prevents the extreme obesity associated with confined cage life and access to unlimited food. At 2 months of age mice were randomly divided into four experimental groups with nine mice in each group. Mice designated for the three calorie restriction groups were switched to a food intake of 9 kcal/day (approximately 60% of the chow eaten when given unlimited access to food) either 1, 4 or 16 weeks prior to tissue collection at 6 months of age. Since our goal was to evaluate the effect of CR on changes in gene expression occurring as early as 1 week on a CR diet, we chose to introduce the CR diet without the gradual acclimatization period that is sometimes used. Mice were sacrificed after a 12-h fast via cervical dislocation. Immediately after cervical dislocation, the abdomen was opened and the adipose tissue and liver placed in liquid nitrogen. Subcutaneous adipose tissue was harvested bilaterally from the inguinal fat pads and visceral adipose tissue from the epididymal fat pads. Plasma was taken at the time of sacrifice for measurement of glucose, insulin and IGF-1. Body weight data are shown in Fig. 1.

### 2.2. Plasma measurements

Glucose was measured in all mice using an Accu-Chek Advantage glucometer (Roche). Plasma insulin and IGF-1 levels were measured in duplicate in 7 or 8 randomly selected mice from each group by the Assay Services Laboratories of the Wisconsin National Primate Research by commercially available RIA kits (Linco EZRMI-13K for insulin, and Diagnostic Systems Laboratories DSL-10-2900 for IGF-1).

### 2.3. Real-time RT PCR

Total RNA was isolated from snap-frozen tissue using the RNA-Bee reagent (Tel-Test, Inc.) and treated with DNase I (New England Biolabs) to remove contaminating DNA. First-strand cDNA synthesis was performed with random hexamer primers using TaqMan Reverse Transcription Reagents (Applied Biosystems). Real-time Pcalorie restriction was performed on the ABI Prism 7700 Sequence Detection System (Applied Biosystems) using SYBR Green Pcalorie restriction Master Mix (Applied Biosystems). Gene-specific primers were designed using Primer Express software (Applied Biosystems) and span intron–exon junctions to avoid amplification of genomic DNA. Specific primer sequences follow:

ACC1 forward 5′-GCCATGTTGAGACGCTGGTC-3′
ACC1 reverse 5′-CTCCTCTATCACAGAGCGGACG-3′
ACC2 forward 5′-CCAGTCTTCCGTGCCTTTGTAC-3′
ACC2 reverse 5′-CTCATCCCTCGCTCTGAACG-3′
FAS forward 5′-GCTGTAGCACACATCCTAGGCA-3′
FAS reverse 5′-TCGTGTTCTGCTTCCAGGATC-3′
PPAR-γ forward 5′-TCGCTGATGCACTGCCTATG-3′
PPAR-γ reverse 5′-TGTCAAAGGAATGCGAGTGGTC-3′

SREBP-1c primer sequences were taken from Yang et al. (2001). Primers for 18S rRNA were purchased from Applied Biosystems. The relative amounts of mRNAs were calculated using the comparative $C_T$ method. 18S rRNA was used as the invariant control. All final values were