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EFFECT OF DIETARY MANIPULATION ON *c-myc* RNA EXPRESSION IN ADIPOSE TISSUE, MUSCLE AND LIVER OF BROILER CHICKENS

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SUMMARY: The effects of dietary restriction on the relative steady state levels of cellular *myc* (*c-myc*) mRNA in abdominal adipose tissue, breast muscle and liver of chickens were determined. Fasting was found to increase *c-myc* RNA expression in adipose tissue ($p<0.01$). This increase returned to normal levels after refeeding. Muscle and liver in fasted birds did not show changes in *c-myc* that differed from controls. Serum concentrations of glucose, triglyceride (TG), free fatty acids (FFA) and insulin-like growth factor (IGF-I) were compared to levels of *c-myc* found in control birds. In adipose tissue, *c-myc* levels were negatively correlated with serum glucose, TG and IGF-I, while in muscle a positive correlation with serum glucose and TG was found. Data suggest that *c-myc* is involved in the metabolic changes occurring in fat cells under fasting conditions.

Nutrient priorities in underfed animals are met in part by a coordinated shift in carbohydrate and lipid metabolism. Reports have shown that the energy balance in chickens results in the cells of certain tissues responding with changes in the synthesis and activity of gluconeogenic, glycolytic, lipogenic and lipolytic enzymes (1, 2, 3). While several hormones and growth factors have been investigated as possible regulators of these cellular responses to nutritional change (4, 5), the molecular mechanisms at the level of gene regulation that mediate these coordinated responses are not understood.

The protooncogene *c-myc* encodes a nuclear phosphoprotein (Myc) that is thought to be involved in the control of cell proliferation (6, 7) and transcriptional regulation of other genes (8, 9). We are interested in nuclear proteins that control gene expression and how these proteins may be involved in the cellular response to nutritional stress. Therefore

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Abbreviations: IGF-I, insulin-like growth factor; TG, triglyceride; FFA, free fatty acids.

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we sought to determine if levels of the cellular (c) protooncogene *c-myc* mRNA are altered in adipose tissue, muscle or liver of chickens subjected to different nutritional regimes.

MATERIALS AND METHODS

Animals, Diets and Experimental Design. A total of 112 three-week-old male broiler chickens (Peterson × Arbor Acres) were randomly assigned to two floor pens (56 birds each) in an open-sided house without any additional light control; each pen represented a control or treatment group. All birds were subjected to a single meal feeding (10:00 AM) for ten days prior to the start of treatments. On day 11, the group of fasted birds were not fed and sacrifice was begun 1, 2, 4, 8, 12 h and 24 h after the start of the normal feeding time. Control birds were fed on schedule and then sacrificed as the fasted birds had been sacrificed, except for one group which was sacrificed at 0 hr. Fasted-refed birds were also restricted from feeding on day 11 and 12 before being refed on day 13 and subsequently sacrificed and tested on the same schedule as above. In all tests, four birds were sacrificed at each time point. Blood samples were collected for serum analysis. Samples of abdominal adipose tissue, breast muscle (*M. pectoralis*) and liver were obtained immediately post-mortem. Tissue samples were wrapped in aluminum foil, frozen by submersion in liquid nitrogen, and stored at -70°C.

Nutritional Metabolite Assays. All metabolites were measured in blood serum. Glucose and TG were determined using commercial diagnostic kits (Sigma Chemical Co.; St. Louis, MO). Free fatty acids were assayed according to Antonis (10); absorbance of the reacted mixture was read at 440 nm using palmitate-albumin as a standard.

IGF-I Radioimmunoassay (RIA). Recombinant human [Thr59] IGF-I (hIGF-I; Amgen Biochemicals, Thousand Oaks, CA) was used for standards and recoveries; iodination was used for assays (11). The chloramine T method of Greenwood et al. (12) was used to iodinate IGF-I. Serum IGF-I was measured according to the protocol of Houseknecht et al. (11) using IGF-I antibody (UBK 487), which was donated by the National Hormone and Pituitary Program, University of Maryland School of Medicine.

RNA Isolation and Analysis. Total RNA was extracted from adipose tissue, breast muscle and liver using the phenol extraction procedure described by Towle et al. (13). Samples of RNA (20 μg) were denatured in a solution of 1 M glyoxal and 50% dimethylsulfoxide (v/v) at 50°C for 60 min (17), and then electrophoresed through a 1.1% agarose gel. RNA transcript size (kb) was determined by comparison with a RNA molecular size marker (#5620SA Bethesda Research Laboratory, MD). Gels were blotted onto nylon membranes (Hybond-N; Amersham, Arlington Heights, IL). DNA-RNA hybridization was carried out at 42°C according to standard procedures (14). Chicken *c-myc* cDNA probe was isolated as an *Eco* RI & *Cla* I fragment (1.4 kb) from plasmid pCMC-Bam supplied by American Type Culture Collection (Rockville, MD). An actin cDNA probe (Oncor, Gaithersburg, MD) was used for comparison in Northern blot analysis. Dot blot analysis was performed by blotting 10 μg of total RNA. Relative expression of *c-myc* mRNA was estimated using a video densitometer (Bio-Rad, Model 620, Richmond, CA).

Statistical Analysis. Data obtained 1, 2, 4, 8 and 12 h after treatment were analyzed using the General Linear Model Procedures and regression analysis of the Statistical Analysis System (15). Significance ($p<0.05$) for treatment, time and interactions were determined by the F-test (15). Linear and quadratic regression analysis was used to fit the statistical models. Significance ($p<0.05$) for the regression analysis was determined by the Student's t test (16). Correlation coefficients were calculated using SAS (15) procedures.

RESULTS AND DISCUSSION

Northern analysis of total RNA from five-week-old birds shows a 2.4 kb transcript corresponding to the known size for *c-myc* (17). Non-specific binding was not found under the conditions employed in the Northern analysis for *c-myc* (data not shown). Levels of *c-myc* mRNA were most affected in adipose tissue of fasted birds which showed levels significantly elevated above controls as evidenced by different ($p<0.01$) Y-intercepts (Fig. 1A). c-myc was also determined at 24 hr in fasted birds and was found to decline to zero time levels (data not shown). This difference in *c-myc* appears to be due to an increase above zero time levels in fasted birds while a decline below zero time values occurred in controls. A small positive trend in slope can be seen in adipose controls during the time interval between feedings (Fig. 1A,B). Thus cyclic feeding of control birds led to a drop of *c-myc* in adipose tissue, which was followed by a slow return to initial levels. On the other hand, a rise in *c-myc* levels in fasted birds was followed by a drop to normal levels by 24 h.

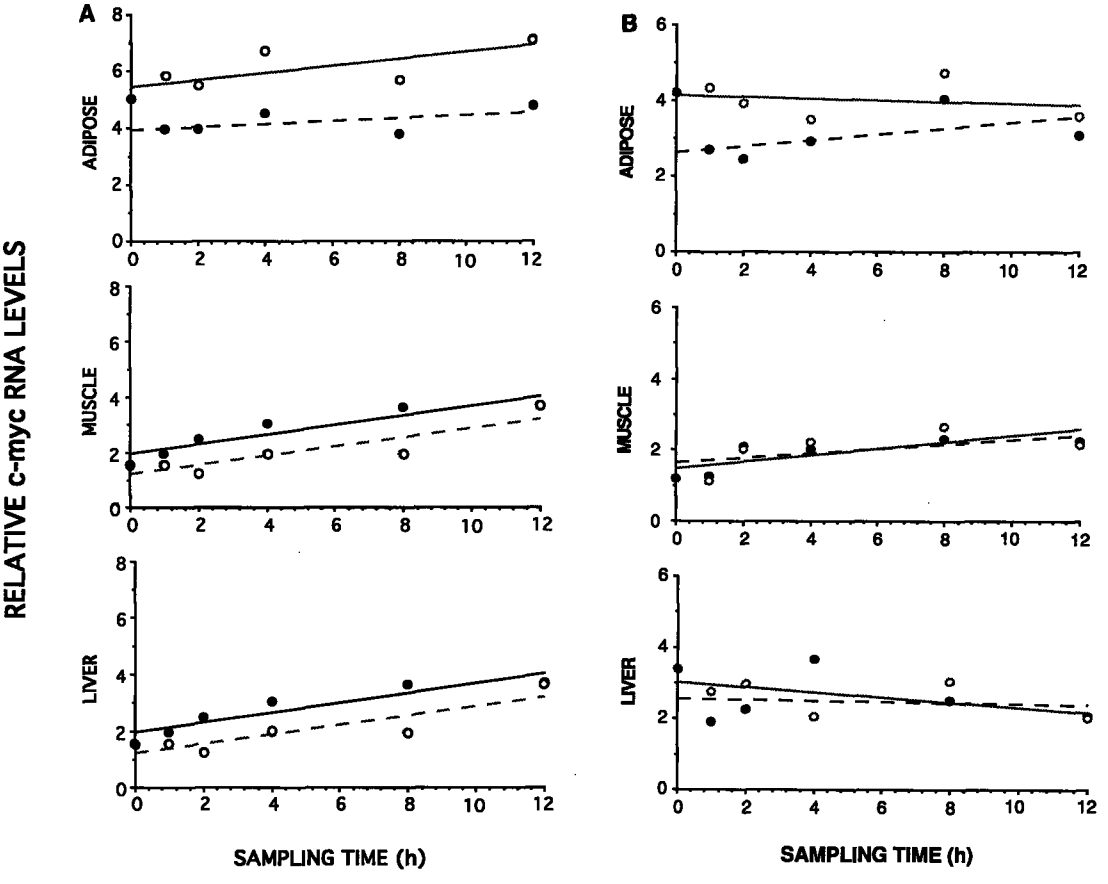

FIGURE 1. Regression analysis for relative *c-myc* RNA expression. Panel A, fasting (o—o) vs control (•—•); Panel B, fasting-refeeding (o—o) vs control (•—•).

Why fasted birds return to normal levels is unknown except that anticipation of the normal feeding schedule may play some role.

Expression of *c-myc* was also determined in fasted-refed (day 13) birds for a period of 12 h immediately after refeeding (Fig. 1B). For both control and test birds, linear regressions were found to fit data for each tissue. In adipose tissue, *c-myc* transcript levels were greater initially in fasted-refed birds than in control birds ($p<0.01$). After about 4 h, this initial abundance was reduced to the control levels. Unlike the rapid and sometimes transient induction of *c-myc* (18, 19), *c-myc* may undergo relatively slow changes in expression in adipose tissue between feedings, or upon refeeding after fasting because the ingestion of food does not result in an immediate change in the cellular environment. No other detectable differences in *c-myc* abundance were observed between controls and fasted-refed birds in muscle or liver.

In muscle there is a trend toward lower levels of *c-myc* in fasted birds but the data did not reach statistical significance. Levels of *c-myc* were found to increase ($p<0.01$) in muscle for both control and test birds over the first 12 h of test (Fig. 1A). This increase, as in the case of adipose tissue, had diminished by 24 h. For liver, the abundance of *c-myc* transcripts remained constant over the test period for control birds but fasted birds showed a initial decrease followed by an increase above control levels. The cyclic behavior in all cases except the liver of fasted birds appeared to be a result of the pattern of the single meal feeding system.

The picture that emerges from our nutritional treatment studies is that fasting increases the development of *c-myc* in adipose tissue and an opposite trend toward lower levels was noted in muscle. Birds that are refed after fasting for two days displayed few differences from controls except that elevated levels of *c-myc* in adipose tissue were lowered slowly to control levels after refeeding. It would appear that when fat cells are not adequately nourished, *c-myc* is increased and when nourishment is replenished after refeeding, *c-myc* returns to normal levels. It is not surprising that adipose tissue is targeted for increased expression of *c-myc* during fasting. Horikawa et al. (20) reported a four- to five-fold increase in *c-myc* expression in rat liver when rats were fed a protein-free diet first and then shifted to a protein-containing diet. Just as the liver is a focus of activity for protein utilization, adipose tissue is a focus during fasting. During fasting, the role of fat tissue is essentially reversed and numbers of enzymes are increased or newly expressed. This kind of cellular "switch" fits the known role of *c-myc* well, as a DNA binding factor that alters the status of a cell in major ways. Under conditions of fasting, muscle is not required to undergo vast change in metabolic activity.

TABLE 1. Serum nutritional metabolites and IGF-I for fasted, fasted-refed and control chickens[a]

Serum components[b]	Fasted	Control	Refed	Control
Glucose	235.9 ± 3.8*	274.9 ± 4.0	315.2 ± 6.0*	271.7 ± 4.2
TG	42.4 ± 1.2*	103.0 ± 5.7	66.3 ± 6.9*	122.6 ± 8.0
FFA	2.0 ± 0.2*	1.6 ± 0.1	1.19 ± 0.1	1.49 ± 0.1
IGF-I	11.1 ± 0.6*	15.7 ± 0.7	7.9 ± 0.4*	14.8 ± 0.7

[a] Values for control birds appear in parentheses directly following the experimental values. Control birds were not fasted. All values are means averaged for the 1, 2, 4, 8 and 12 h sampling times.

[b] Values are in mg/dl for glucose and TG, meq/l for FFA, and ng/ml for IGF-I.

* Means the statistical difference ($p < 0.05$) between treatment and control groups; for each value, n=20.

Serum metabolites were evaluated in test and control birds to confirm conditions of fasting and to see if correlations between *c-myc* expression in tissues and serum metabolites could be found. Table 1 summarizes metabolite mean values during the test period. Cyclic behavior in glucose was observed during testing in control birds. For fasting birds, glucose, TG and IGF-I showed lower mean values than controls ($p < 0.01$), while the values for FFA were significantly ($p < 0.05$) higher than controls. During refeeding, mean glucose levels in refed birds increased above those of the control birds (Table 1). Serum TG and IGF-I remained significantly below control levels, and no significant difference could be found in FFA.

Data for test and control birds were examined to see if correlations in individual birds could be established. In test birds, correlations between *c myc* and serum metabolites or IGF-I could not be established; however, in control birds correlations were found. The analysis combined data from both fasted and fasted-refed studies of control birds since their treatment was the same. Correlation coefficients between measures of *c-myc* and RNA expression in adipose tissue, muscle and liver and serum metabolites and serum IGF-I concentrations were determined (Table 2). Serum concentrations of glucose ($p < 0.05$), TG

TABLE 2. Correlation coefficients between measures of relative *c-myc* levels in tissues and serum concentrations of nutritional metabolites nd insulin-like growth factor-I (IGF-I) in individual non fasted control chickens

	Serum Components[a]			
Tissue *c-myc*	Glucose	TG	FFA	IGF-I
Adipose	-0.39*	-0.59**	-0.02	-0.39*
Breast muscle	0.46*	0.45*	-0.02	0.31
Liver	0.26	0.16	0.02	-0.05

[a] Abbreviations: TG, triglyceride; FFA, free fatty acids.

* $p < 0.05$.

** $p < 0.01$.

($p<0.01$) and IGF-I ($p<0.05$) were negatively correlated with *c-myc* expression in adipose tissue. In contrast, serum concentrations of glucose and TG were positively correlated ($p<0.05$) with *c-myc* RNA expression in muscle. Thus, the natural variation in serum metabolites between individual birds, under control conditions, produced strong negative correlations for adipose tissue and an opposite positive correlation in muscle. This opposite trend was also observed in the fasting treatments between adipose and muscle. Conclusions are difficult to draw from the data correlating metabolites and *c-myc*. No correlation could be found in the test birds, indicating that *c-myc* is uncoupled from serum metabolites. The correlation found in controls birds may be due to regulator factors that coordinately regulate metabolites and *c-myc*.

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