EFFECTS OF DIETARY RESTRICTION ON INSULIN RESISTANCE IN OBESE MICE

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ABSTRACT:

In many cases, development of insulin resistance has been linked to obesity and may contribute to mechanism of aging. The role of diet, irrespective of degree of obesity, in modulating insulin resistance and development of age degeneration disease remains uncertain. Lowered blood glucose levels are commonly associated with diet restriction (DR), which is an intervention shown to successfully retard aging and age associated disease. The effects of DR on blood glucose and insulin resistance were measured in yellow obese (A^y/A), lean black (a/a) mice and in another common inbred strain (B6C3F1) (at three different ages). The yellow obese mice become diabetic as a result of an insulin receptor defect which is not clearly understood. Insulin responses and radioinsulin binding were assayed in yellow obese and lean black mice fed either *ad libitum* (AL) or DR diets (YAL, BAL, YDR and YAL, respectively) at four different circadian intervals. The B6C3F1 controls were fed either AL (CAL) or DR (CDR) and measures were made at six circadian stages and three different ages. Within 23 days, DR produced a significant loss in body weight and a time-dependent 22-55% reduction in basal blood glucose levels in the yellow obese mice. Additionally, exogenous insulin produced circadian stage dependent (at the time of food intake) reductions in blood glucose in the YDR animals that were not present in YAL animals. ¹²⁵I-Insulin binding in liver was increased nearly 2-fold in YDR and BDR

mice during the time of day that animals were active and eating. ¹²⁵I-Insulin binding was two-fold-higher in CDR mice at 4, 12 and >24 months of age. Binding decreased as a function of age in both the CAL and CDR animals. However, even in the >24 month group the CDR animals were found to have levels of binding that were as high as those found in younger CAL liver. The mechanism of action appears to be through resolution of insulin resistance by modulating an insulin receptor defect.

INTRODUCTION

It is currently accepted that obesity is associated with an array of age associated pathologies (Hsueh & Buchanan, 1994; Grundy, 1998), leading to major health problems, especially in older populations and obesity is increasingly thought of as accelerated aging (Ilarde & Tuck, 1994; Falkner & Michel, 1999). Insulin resistance has been identified as one of these common disorders, as it has often been associated with obesity (Smith, 1994). Whether insulin resistance (non-insulin-dependent diabetes mellitus: NIDDM) is possibly a cause or an effect of obesity was not clear (McGarry, 1994; Masoro, 1996); but, a case was been made for obesity as a major factor of insulin resistance (Cooney & Storlien, 1994; Golay & Felber, 1994; Scheen & Luyckx, 1999). It is generally accepted that diet and bodyweight reduction are central to control of NIDDM, with a reduction in calories as a primary consideration (Ilarde & Tuck, 1994). However, most often emphasis has been upon diet constituency where it is attempted to increase the carbohydrate-fat ratio (Smith, 1994). Thus, the role of diet and diet restriction (DR) in modulating insulin sensitivity in obesity has not been totally clarified (Smith, 1994).

Obesity has been characterized by distinct patterns of hormonal changes such as hyperinsulinemia, alterations

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in adrenocortical activity and sex steroid secretion and binding (Kopelman, 1994; Johannsson & Bengtsson, 1999). Also, the hyperinsulinemia and hyperglycemia associated with NIDDM have been well documented for many years (Golay & Felber, 1994; Meehan, et al., 1994; DeFronzo, 1999). Furthermore, hyperinsulinemia and hyperglycemia, have been proposed as an underlying problem of obesity and NIDDM (Hodge & Zimmet, 1994; Jha, 1999). The role of insulin receptor in NIDDM has not proven to be consistent, since obesity and insulin resistance may be due to changes in the level of the receptor, changes in binding affinity or post-receptor events (Shafrin, 1996; Alemzadeh, et al., 1996; Hotamisligil, 1999).

Diets low in available calories, but with adequate levels of essential nutrients, produce extended life-span in rodents when compared to *ad libitum* (AL) fed animals (Ebling & Koivisto, 1994; Ilarde, & Tuck, 1994; Smith, 1994; Masoro, 1996; Shafrin, 1996; Weindruch, et al., 1997; Frame, et al., 1998; Roth, et al., 1999). Several physiologic and metabolic parameters which may be directly related to regulation of blood glucose and other metabolic processes have been shown to be altered by DR (Duffy, et al., 1989; Feuers, et al., 1989; Weindruch & Walford, 1988; Frame, et al., 1998). Additionally, when animals were placed on diets which were reduced in calories by 40%, body weights were reduced proportionally (Weindruch and Walford, 1988). It was also demonstrated that the reduction in body weight was accompanied by significant reduction in blood glucose (Feuers, 1991), and circulating insulin and changes in levels of a number of other hormones such as the glucocorticoids (Leakey, et al., 1989). In addition, the insulin receptor has also been shown to be upregulated by as much as two-fold by DR (Feuers, et al., 1991). Each of these parameters was represented by circadian change with food intake as a primary synchronizer (Duffy, et al., 1989; Feuers, 1991; Leakey, et al., 1989).

The ability of DR to improve the regulation of blood glucose in an obese, animal model has not been studied. One goal of this investigation was to determine if obese mice were insulin resistant, and if so, determine if DR might relieve this condition. A further purpose of this study was to determine if improved glucose regulation through increased insulin sensitivity by DR may play a role in the mechanism through which DR extends maximum achievable lifespan.

MATERIALS AND METHODS

Weanling (C57BL/6Nctr x YS/WffC3Hf/Nctr-A^{vy}) F₁ hybrid mice were used in this experiment. Approximately half the litter mates were yellow (n=55) and develop obesity and half the litter mates were black (n=65) and remained lean. Animal allocations are as shown in Table 1. The mice are standardized to a light schedule where the lights went off at 1600 hr and came back on at 0400 hr. Animals were fed between 1500 hr and 1600 hr each day.

Table 1: Animal Allocations

Genotype	Total/diet	Time-of-Day			
		0600	1200	1800	2400
55A ^{vy} /a (yellow)	27 AL	6	7	7	7
	28 DR	7	7	7	7
65 a/a (Black)	32 AL	8	8	8	8
	33 DR	8	8	8	9

AL = *Ad Libitum*

DR = Diet Restricted

Standard NIH diets were used throughout the experiment. Food consumption was measured three times a week and from these data a daily consumption average was calculated. This was done until the animals were 14 weeks old. At week 15, approximately 50% of the yellow and approximately 50% of the black mice were given 90% of the total daily consumption of the AL population using the standard NIH-31 PCR diet that was vitamin supplemented. Diet restriction resulted in a reduction of all nutrients except vitamins which were fortified at 1.67 times the concentration of the NIH-31 standard AL diet formulation. At week 16, the total food given to the DR groups was further reduced to 75% of that given to the AL group. A week later and for the next 23 days, the total food given to the DR group was reduced to 60% of that given to the AL group.

After 23 days on the 60% diet, the yellow and black AL and DR mice were divided into groups of 6-9. Beginning at 1800 hr, each group of mice was injected subcutaneously with 0.15 ml of a saline solution containing six units of regular, unmodified beef insulin and (obtained from ICN Incorporated) 0.4 mCi ¹²⁵I-insulin. Just prior to injection, a drop of tail blood was obtained for basal glucose determination using the one-touch glucose analyzer (Lifescan, Johnson and Johnson). Thirty minutes later, another blood sample was taken and the animals were sacrificed by CO₂ anesthesia. The left lobe of the liver and the left epididymal fat pad were rapidly excised and weighed. Counts per minute per gram of sample were measured in a Packard Auto-Scintillation Spectrograph standardized for [¹²⁵I] assays (Feuers, et al., 1990, a and b). Comparisons of insulin binding between the various groups was done by ANOVA, and by cosinor regression (Halberg, et al., 1972).

RESULTS

Our past experience has shown that even 3-7 days was sufficient to cause reversal in blood glucose levels when diets were switched from AL and DR (or visa versa) using an inbred mouse strains (unpublished data). Additionally, we have seen that short-term DR was sufficient to upregulate the insulin receptor in inbred strains which weighed significantly less than their AL counterparts (Feuers, et al., 1990a; Feuers, et al., 1990b; Feuers, et al., 1991). In this present study the yellow and

black mice lost significant amounts of weight within the 23 day period of restriction. Yellow mice weigh 25% more than their black counterparts, and they lost more than 11% of their body weight during the relatively short time on DR (Table 2). The black mice lost approximately 15% of their total body weight during the DR period. Total weight loss was similar for both the yellow and black mice. Blood glucose levels were reduced significantly in the YDR mice at all times tested (Figure 1, Panels A and B), but not in YAL mice. The declines were most dramatic during the mid-dark span. Blood glucose reached maximums in the YAL mice during the mid-dark span, and amplitude of circadian change (cosinor, $P < 0.001$) was significant since the glucose levels declined by 48% during the mid-light (Table 3). Acrophase (or calculated time of highest blood glucose) for all other mice was determined to be at mid-dark after the animals eat (Table 3). The temporal decline in blood glucose of YAL mice correlates with the cessation of food consumption in these nocturnal rodents (Duffy, et al., 1989; Duffy, et al., 1990a; Duffy, et al., 1990b; Duffy & Feuers, 1991a; Duffy, et al., 1991b; Feuers, 1991). The circadian rhythm found for YDR mice was significant ($P = 0.05$), but the amplitude of the rhythm was greatly reduced and similar to both BAL and BDR mice (Table 3, and Figure 1, panels B, C and D)]. Blood glucose levels were not different when BDR rodents were compared to the BAL mice, and the circadian rhythms (cosinor, $P < 0.05$) were similar for the BAL mice (Table 3, and Figure 1, panels C and D).

Table 2: Body weight of Dietary Restricted and Ad libitum Fed Lean Obese Mice

Animal/Diet	Weight (gm)
YAL	54.6 ^d ± 2.1
YDR	48.2 ^a ± 1.2
BAL	41.3 ^b ± 0.9
BDR	33.3 ^c ± 0.9

YAL = yellow obese, ad libitum fed, YDR = yellow obese, diet restricted, BAL = black lean, ad libitum fed, BDR = black lean, diet restricted

When the YAL animals were given exogenous insulin, no significant reduction in blood glucose was seen, and

circadian rhythms were also similar (Table 3, and Figure 1, panels A). However, when exogenous insulin was given to the YDR mice, blood glucose levels were reduced at each time-of-day, with the reduction being 33-41% (Figure 1, panel B). The circadian rhythms for blood glucose of YDR before and after insulin were different only for mesor which is the cosinor calculated 24-hr mean (Table 3). Exogenous insulin affected BAL and BDR mice similarly, with the response being slightly larger in BDR mice. Blood glucose reductions were between 38 and 67% in BDR and ranged from 39-51% in BAL mice (Figure 1, panels C and D). The responses to exogenous insulin were similar for YDR, BAL, and BDR mice (Figure 1, panels B, C and D).

The circadian responses to ¹²⁵I-insulin binding in liver for YAL and BAL mice were nearly opposite (Figure 2, panel A). Maximum binding (acrophase) occurred during the late-dark span when blood glucose was highest in YAL mice, while acrophase was later in the BAL mice (at the time of transition from light to dark, data not shown). Additionally, the mesor value for total binding, and amplitude of the rhythm were higher in BAL mice (Figure 2, panel A). When the yellow mice were placed on DR for 23 days, total binding was significantly increased at 1400 and 2000 hr (33 and 25% respectively). This was at the time of transition from light to dark when the pattern of binding was similar to that of BAL mice (Table 4, figure 2). In the BDR mice, binding was significantly increased by DR at 0800 (53%) and 2000 (46%) hr (Figure 2, panel B). In another earlier experiment using another (common) control strain of mice we found that binding to liver was also higher in normal B6C3F1 (CAL or CDR) animals at 4, 12, or >24 months of age (Figure 4, from Feuers et al., 1990b). Total binding decreased as a function of age in both AL and DR B6C3F1 mice, but was always higher in the DR animals. Additionally, DR animals were found to have more significant circadian rhythms for total binding and the timing of acrophase was closely linked to patterns of food consumption. Further, blood glucose reductions were more pronounced in response to exogenous insulin in DR animals at all ages (data not shown, Feuers, et al., 1990b).

Table 3: Cosinor Regression Analysis of Blood Glucose as Effected by Dietary Restriction in Lean and Obese Mice

Animal/Diet/Status	P Value	Mesor	Amplitude	Acrophase
YAL-B	0.482	170.3 ± 20.8	53.3 ± 29.3	0052
YAL-PI	0.310	153.5 ± 11	47.7 ± 15.6	2327
BAL-B	0.488	135 ± 6.5	16.4 ± 9.2	1841
BAL-PI	0.115	68.5 ± 0.5	6.1 ± 0.7	0122
YDR-B	0.128	110 ± 1	11 ± 1.4	2000
YDR-PI	0.544	67.3 ± 0.3	13.6 ± 8.8	2033
BDR-B	0.333	118.5 ± 4.5	18 ± 6.3	2012
BDR-PI	0.554	52 ± 8.5	18.1 ± 12	2057

YAL = yellow obese, ad libitum fed, YDR = yellow obese, diet restricted, BAL = black lean, ad libitum fed, BDR = black lean, diet restricted

B = Basal or pre-insulin blood glucose, PI = Post insulin blood glucose, P = fit to cosinor regression

Mesor = cosinor calculated 24 hr mean, Acrophase = cosinor calculated highest anticipated value

Amplitude = Cosinor calculated difference between highest and lowest anticipated values

Figure 1: Basal and post insulin blood glucose level in ad libitum (AL) or dietary restricted (DR) yellow and black mice at different times of day. Panel A= YAL mice, Panel B= YDR mice, Panel C= BAL mice, and Panel D= BDR mice. Open marker = basal blood glucose and closed marker = post insulin glucose. Black bars on x-axis indicate time lights were off.

There were no circadian rhythms or differences noted for ^{125}I -insulin binding to epididymal fat in either YAL or BAL mice (Figure 3, panel A). The only effect of DR was found in BDR mice during the early dark-span, when binding increased by 83%; however, the standard deviation was considerably larger (Figure 3, panel B). The increased binding at 2000 hr did produce a significant circadian rhythm (data not shown). In femoral muscle, binding was not different among the various groups of mice at the various times of day, and there was no upregulation of the insulin receptor (data not shown). This was possibly a result of increased variability since standard deviation was considerably higher in femoral muscle than in liver or fat.

DISCUSSION

The significantly higher body weights found in YAL animals as compared to their BAL counterparts correlated favorably with blood glucose levels. Thus, obesity was associated with hyperglycemia that occurred during the dark-span when these nocturnal animals were eating. The lack of response to exogenous insulin in the YAL mice was also associated with obesity since the

Figure 2: ^{125}I -Insulin binding to liver (CPM/gram) in ad libitum (AL) or dietary restricted (DR) yellow and black mice at different times of day. Panel A = ^{125}I -insulin binding in YAL and BAL mice, open circle = YAL mice, open triangle = BAL mice. Panel B = ^{125}I -insulin binding in YDR and BDR mice, closed circle = YDR mice, and closed triangle = BDR mice. Black bars on x-axis indicate time lights were off.

Figure 3: ^{125}I -Insulin binding to fat (CPM/gram) in ad libitum (AL) or dietary restricted (DR) yellow and black mice at different times of day. Panel A = ^{125}I -insulin binding in YAL and BAL mice, open circle = YAL mice, open triangle = BAL mice. Panel B = ^{125}I -insulin binding in YDR and BDR mice, closed circle = YDR mice, and closed triangle = BDR mice. Black bars on x-axis indicate time lights were off.

BAL mice responded to exogenous insulin. The apparent insulin resistance found in the YAL mice was not present in the BAL mice. It was interesting that glucose levels declined to near normal amounts during the light-span in the YAL animals. This suggested that some level of regulation of blood glucose occurred in YAL mice.

When the YAL animals were placed on DR, and as body weight was declining, their blood glucose levels dropped to levels similar to those seen in this BAL animals. Furthermore, the YDR and BAL animals responded similarly to exogenous insulin. This suggests that there was either no insulin resistance or greatly reduced resistance in the YDR mice. Therefore, even a relatively short period of DR resolved hyperglycemia and insulin resistance in the yellow obese mice. There

Table 4: Cosinor Regression Analysis of Liver and Fat 125 I-insulin Binding in Dietary Restricted and Ad Libitum Fed Lean and Obese Mice

Animal/ Diet	Tissue	P Value	Mesor	Amplitude Acrophase (hrs)	
YAL	L	0.18	1957 $\pm$ 28	212 $\pm$ 39	0600
BAL	L	0.06	3008 $\pm$ 32.5	746 $\pm$ 46	1631
YDR	L	0.29	2469 $\pm$ 87	413 $\pm$ 123	1346
BDR	L	0.88	4765 $\pm$ 1259	944 $\pm$ 1781	1617
YAL	F	0.85	1232 $\pm$ 90	78 $\pm$ 127	0440
BAL	F	0.03	1060 $\pm$ 3	176 $\pm$ 5	0440
YDR	F	0.69	1166 $\pm$ 87	127 $\pm$ 123	1138
BDR	F	0.45	2795 $\pm$ 1009	2856 $\pm$ 1427	2000

YAL = yellow obese, ad libitum fed

YDR = yellow obese, diet restricted

BAL = black lean, ad libitum fed

BDR = black lean, diet restricted

Mesor = cosinor calculated 24 hr mean, Acrophase = cosinor calculated highest anticipated value

Amplitude = Cosinor calculated difference between highest and lowest anticipated values

L = Liver, F = Fat

P = fit to cosinor regression

was a slightly stronger increase in response to exogenous insulin in the black mice after they had been placed on DR, since the decrease in blood glucose was about 10% more in the BDR than in the BAL mice. This may suggest a slight insulin resistance in BAL or increased insulin sensitivity in the black mice as a result of DR. Since the level of blood glucose in the YDR mice decreased to levels similar to that of the BAL mice in response to exogenous insulin, DR resolved insulin resistance in the yellow mice to a point where the YDR mice were similar to the BAL mice. However, the blood glucose values were not reduced to the levels seen in BDR animals. These observations support the hypothesis that there may be more than one mechanism of insulin resistance within these animals. One of these was resolved by DR in the yellow mice, and another that was resolved in the black mice during the period of DR. The mechanisms are not known, but may be at the level of the receptor and/or they may be post-receptor defects. If more than one mechanism exists, further time on DR or higher doses of insulin may allow the yellow mice to achieve additional insulin sensitivity by resolving the second insulin resistance. If only a single mechanism exists, further time on DR may produce reductions of blood glucose in response to insulin that are similar to those of the black mice on DR.

DR did restore circadian rhythms and the regulation of glucose levels in the yellow mice that which were similar to those seen in the normal black mice. This suggests that endocrine regulation and the response to hormones such as insulin are normalized by DR, and this may indicate that hyperinsulinemia is resolved. It has been previously shown that DR yielded decreased levels of circulating insulin which was correlated to decreased blood glucose, and the upregulation of the insulin receptor (Leakey, et al., 1989). Each of these parameters were characterized by prominent circadian rhythms, the

Figure 4: 125 I-Insulin binding to liver in young, middle age and old ad libitum fed (AL) or dietary restricted (DR) B6C3F1 male mice at different times of day. Panel A = young (4 months), Panel B = Middle age (12 months) and Panel C = old (>24 months). AL = closed marker, DR = open marker. Black bars on x-axis indicate time lights were off.

phasing of which was correlated to food intake. These factors were correlated to the DR restored circadian rhythms in yellow obese and black mice.

The degree of insulin binding was time-of-day dependent and tissue specific suggesting increased affinity for receptor at times associated with food intake. This supports the notion that total receptor number was not changed since upregulation (followed by down-regulation) during the 24-hr span would be required. Additionally, the binding was more robust in a normal inbred strain (B6C3F1 mice) at all ages (except old AL mice) just after food ingestion. (Feuers, et al, 1990a and b). These observations further support the notion that the lack of regulation of glucose levels in yellow obese mice may be, in part, due to a receptor and post-receptor insulin resistance and that mechanisms of insulin resistance may be multifaceted.

No increase of binding in fat was seen in YDR mice as compared to YAL mice and binding was increased at only one time-of-day in the BDR mice. DR did not improve insulin binding in yellow obese mice. However, the length of time on DR may not have been sufficient, and weight loss may not have been adequate to trigger this effect. Alternatively, binding in fat may be maximally upregulated in YAL mice, and this could contribute to

efficiency of fat storage. Weight loss and loss of fat mass may affect this situation. Indeed, time-of-day dependent changes were observed in the thinner BDR mice, and this suggests that DR does have some affect on the insulin receptor in fat.

When the ^{125}I -insulin binding level was determined in liver, we found a significant increase (but not at all times-of-day) in both YDR and BDR relative to YAL and BAL, respectively. Therefore, there was an increase in insulin sensitivity and/or binding affinity with a resultant reduction in blood glucose and improved circadian regulation of glucose levels. However, these increases in binding were not as large as we saw in normal inbred strains where DR increased binding equally at all time-of-day for young, middle aged, and old mice (Feuers, et al, 1990a and b). In the normal AL mice, binding was lower than in their DR counterparts, and this was true at all ages. Even though, binding did go down with age in both AL and DR mice. However, binding was as high in DR animals >24 months of age and similar to that seen in AL animals at 4 months or 12 months of age. Therefore, DR increases binding, reduces insulin resistance, and possibly improves post-insulin receptor defects, thereby improving metabolic efficiency at all ages.

The relationship of obesity to development of insulin resistance is clear. However, the role of diet in modulating insulin resistance in obesity has not been totally delineated (Smith, 1994). It is now clear that, in yellow obese mice, DR effectively reduces insulin resistance, and coupled with weight loss, DR may modulate basic mechanisms that reduce the rate of development of age associated degenerative disease. The concomitant weight loss, improved insulin sensitivity, and temporal regulation of blood glucose in the presence of reduced circulating levels of insulin may significantly contribute to improved efficiency of enzyme systems of intermediary metabolism (Feuers, 1991) and suggests a potential for reducing or eliminating many of the pathologies associated with obesity and aging by DR. These factors may be part of a mechanism by which DR increases maximum achievable lifespan.

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