REVISTA ESPAÑOLA DE FISIOLOGIA, 36, 255-264. 1980

Effect of Diabetes and Protein Malnutrition on the Rate of Muscle Protein Breakdown in Rats

S. Santidrián *, V. R. Young and H. N. Munro

Massachusetts Institute of Technology
Department of Nutrition and Food Science
Cambridge, Ma. 02139 (USA)

(Received on September 28, 1979)

S. SANTIDRIAN, V. R. Young and H. N. MUNRO. *Effect of Diabetes and Protein Malnutrition on the Rate of Muscle Protein Breakdown in Rats.* Rev. esp. Fisiol., 36, 255-264. 1980.

Male streptocozin diabetic rats were fed *ad libitum* in two diets, one a control, adequate in protein and energy, and another, depleted in protein, but adequate in energy. Within each one of these dietary groups, three hormone-treated groups were made as follows: rats receiving vehicle, or 0.25 or 0.50 I.U. insulin/100 g body weight/day i.p. for 21 days. A fourth group of intact rats, receiving vehicle injection, was included as a control. Every day urine excretion was collected for urea-N and 3-methylhistidine (3-Mehis) determination. Body weight and food intake were recorded daily. At the end of the experiment, all animals were sacrificed, and a sample of blood was taken for plasma insulin assay. Liver, as well as gastrocnemius, soleus and extensor digitorum longus muscles were excised and weighed.

Results showed that diabetic animals had a reduced body weight gain, although the food intake was elevated in all groups, as compared to the intact rats. Gastrocnemius and soleus muscle weights were, respectively, reduced and increased in the diabetic animals fed the low-protein diet. Urea-N output was elevated in all groups fed the control diet, but a marked reduction was observed in the protein depleted rats. A reduction in 3-Mehis output was displayed by the diabetic animals, specially those fed the low-protein diet. The results of this experiment showed that in streptocozin diabetic rats there was a reduction in the rate of myofibrillar protein breakdown, specially marked when fed a protein depleted diet.

* Author's address: Santiago Santidrián, Ph. D.
Massachusetts Institute of Technology
Department of Nutrition and Food Science
Room 56-320
Cambridge, Ma. 02139
USA.

After April 1981: Departamento de Fisiología (Sección de Nutrición). Universidad de Navarra. Pamplona (España).

The synthesis and catabolism of carbohydrate, protein and fat are deeply influenced by many hormones. Insulin is one of these hormones. It affects protein metabolism lowering the blood amino acids level, parallely with reduction in blood sugar (18). It enhances the incorporation of amino acids into proteins in isolated tissues (4, 28) and it acts in several

hepatic enzymes involved in glycolysis and gluconeogenesis (20, 33). The fact that the blood sugar is lowered by insulin injection in man and animals has focused the attention on the effects this hormone has on carbohydrate metabolism, and many attempts to explain this blood sugar lowering have been made (6). In searching for the primary action of insuline, various investigators have examined its effects on RNA synthesis (21, 35).

The untreated state of diabetes is commonly regarded as being of a catabolic nature (7, 26). *In vivo* studies have demonstrated that alloxan-diabetic animals have a reduced rate in protein synthesis as well as a decreased glucose uptake (39). Although the effects of insulin in muscle are quite apparent, it has not been shown that increased muscle protein degradation rates are not responsible for some of the observed catabolic states on diabetes. On the other hand, since the effects of insulin are seen in both protein metabolism and carbohydrate metabolism, it was felt that dietary restriction of one of these classes of nutrients might accentuate or remedy the catabolic effects of diabetes. Previous results obtained in our laboratory (still unpublished), using an adequate protein diet, have led us to investigate the effect of a protein deficient-diet on muscle protein breakdown in diabetic rats receiving different hormonal replacement doses.

In order to assess the possible role of increased protein degradation in the net catabolism of diabetes, this study utilizes the urinary excretion of 3-methylhistidine (3-Mehis), an amino acid derivative present in myofibrillar protein (13), as an index *in vivo* of muscle protein breakdown, as noted by YOUNG (36) and YOUNG and MUNRO (38). It has been demonstrated that this amino acid is quantitatively excreted when it is released during the breakdown of protein muscle tissue (14). Through the use of this parameter, separation of synthesis and degradation are possible. This amino acid derivative itself is a simple and an accurate means of measuring muscle protein breakdown *in vivo* in rat and humans under various conditions and treatments (38). It can be concluded that the diabetes status is very sensitive to the protein content in the diet, and that there is a reduction in muscle protein breakdown in the diabetic animals, especially in those undergoing a protein malnutrition.

Materials and Methods

Animals and experimental design. Diabetic rats (65 mg of streptozotocin/kg body weight, from Sigma) and intact male Sprague-Dawley rats about 125 g body weight (Charles River Breeding Laboratories, Wilmington, Mass.) were housed in individual metabolic cages, where a purified agar-diet containing 18 % lactalbumin, was offered *ad libitum* for one week. Then, the animals were weighed and divided into two groups of 20 rats each, so that the mean body weight within each group was about 160 g. They were the following: control (fed on an adequate protein and energy diet, containing 18 % lactalbumin), and low-protein (fed on a protein-deficient diet, containing 1 % lactalbumin, but isocaloric in relation with the control one). Both diets were provided to the animals *ad libitum*, during 21 days. Composition of the diets are shown in table I. Within each one of these groups, four subgroups of 5 rats each, according to the hormonal treatment, were made. They were the following: intact control, receiving vehicle injection (a 0.9 % NaCl solution), diabetic, receiving vehicle injection, and diabetic receiving 0.25 or 0.50 I.U. of insulin/100 g body weight/day. Every day each rat received a single injection of either vehicle or insulin (Sigma). Injections were given intraperitoneally for 21 days. On the 21st day, all rats were killed by decapitation. All available blood was then collected, the plasma was separated by centrifugation, and stored at

—20° C for measurement of insulin concentrations. Immediately, liver, as well as gastrocnemius, soleus and extensor digitorum longus muscles were excised from rear legs by carefuly dissection, and weighed, following the method given by ARVILL *et al.* (1) and then considered under the metabolic criteria exposed by GOLDBERG (10). Body weight changes and food intake were recorded daily for each rat.

Determination of urinary constituents. Aliquots of the daily urine collection within each group were centrifuged and used for the determination of urea nitrogen and 3-methylhistidine. Concentrations of 3-Mehis were determined on a Beckman 121 Automatic Amino Acid Analyzer, as previously described by BILMAZES *et al.* (3) and HAVERBERG *et al.* (14), after prior hydrolisis of the N-acetyl derivative with 2 N HCl in a boiling water bath for 2 hours and subsequent desalting on a cation exchange column (Dowex AG50-X8) followed by stepwise elution with 2.0-2.5 N HCl of the acidic and neutral, and with 4.0-5.0 N HCl of the basic amino acids. This last eluate, containing the basic amino acids, was dried in a rotatory evaporator and the sample reconstituted with citrate buffer, pH 2.2, before application to the amino acid analyzer. Urea nitrogen was determined by the method of FOSTER and HOCHOLZER (9).

Plasma Hormone Assays. After obtaining the blood from the decapitation wound, approximately 24 hours after the last insulin injection, plasma insulin levels were determined using the Radioimmunoassay Kit (double antibody) provided by Bio-Ria, Lousville, Ky. USA.

Statistical Procedures. Statistical evaluations were carried out by conventional one — and two — ways analysis of the variance.

Results

Effect of treatment on body weight, organ weight and food intake. Figure 1 shows changes in body weight of the groups of rats during the experiment. All values presented are the mean for five rats. During the course of the experiment, all rats fed on the protein-deficient diet failed to grow significantly, as compared to those fed on the control diet ($p < 0.02$). None of the diabetic groups showed growth compared to that demonstrated by the intact rats on the control diet. Only those rats receiving 0.25 I.U. of insulin demonstrated a parallel pattern of growth with respect to the diets, compared with the intact rats. Rats receiving 0.50 I.U. of insulin and fed on the control diet showed a significant decrease in body weight ($p < 0.02$), compared to that exhibited by the intact rats. Table II shows the observed weights of liver and muscles at the completion of the experiment, as well as the food intake of the different groups. No differences were noted in the

Table I. *Diet constituents: entries are g % dry diet.*

Both control and low-protein diets were provided *ad libitum*. The mineral mix was purchased from General Biochemicals, Chagrin Fall, Ohio, USA. Composition of mineral and vitamin mixtures is as described by ROGERS and HARPER (29). Choline was added to the diet as an aqueous solution containing 1 g of choline hydrocloride/5 ml. The corn oil was obtained from Wesson Oil Sales Co., Fullerton, Calif.

Component	Control diet	Low-Protein diet
Dextrine	44.2	55.5
Sucrose	22.1	27.8
Lactalbumin	18.0	1.0
Mineral Mix	5.0	5.0
Vitamin Mix	0.5	0.5
Choline	0.2	0.2
Corn Oil	10.0	10.0
Agar	4.0	4.0
Water	100.0	100.0

Table. II. *Liver (g/100 g b. wt.), muscles weight (mg/100 g b. wt.), food intake (g of diet/100 g b. wt.) and plasma insulin levels (μU/ml) of intact and diabetic rats receiving or not insulin replacement (0.25 or 0.50 I.U./100 g b. wt./day), and fed* ad libitum *on an adequate control diet (18 % lactalbumin) or low-protein diet (1 % lactalbumin) for 21 days.*

Entries are mean ± S.E.M. for five rats. E.D.L. means extensor digitorum longus muscle. Muscles were removed from the rear legs. Rats were killed by decapitation 24 hours after the last injection of either vehicle or insulin. Blood was collected directly from the decapitation wound, and the plasma was assayed for insulin subsequent to separation. Due to the smaller body size of the Diabetic + veh. animals, only a small amount of plasma could be collected and had to be pooled. For this reason, no statistic is presented. Vehicle consisted in a 0.9 % NaCl solution.

Diet	Insulin treatment	Liver wt.	Gastrocnemius wt.	Soleus wt.	E. D. L. wt.	Food intake	Plasma Insulin levels
Control	Intact + veh.	5.00 ± 0.27	1,200 ± 35	76.3 ± 2.4	92.1 ± 4.7	15.3 ± 4.7	85.59 ± 2.52
	Diabetic + veh.	4.65 ± 0.20	1,210 ± 30	76.2 ± 2.7	91.5 ± 5.2	22.5 ± 1.4[a]	7.20
	0.25 I. U.	4.63 ± 0.21	1,210 ± 28	72.7 ± 2.9	92.2 ± 6.2	21.2 ± 1.0[a]	25.00 ± 5.68[a]
	0.50 I. U.	4.71 ± 0.24	1,190 ± 27	72.1 ± 3.7	90.1 ± 4.3	27.9 ± 1.4[a]	10.00 ± 1.20[a]
Low-Protein	Intact + veh.	4.50 ± 0.15	1,200 ± 40	88.1 ± 4.1[b]	94.3 ± 5.3	12.3 ± 0.4	23.75 ± 9.44[b]
	Diabetic + veh.	4.71 ± 0.25	1,000 ± 30[ab]	84.2 ± 3.9[b]	90.7 ± 6.8	28.0 ± 1.0[ab]	6.50
	0.25 I. U.	4.72 ± 0.29	1,100 ± 36	79.9 ± 2.1[ab]	90.5 ± 4.9	28.2 ± 0.8[ab]	23.50 ± 5.68
	0.50 I. U.	4.45 ± 0.18	1,100 ± 38	86.1 ± 5.2[b]	90.1 ± 3.6	27.8 ± 2.4[a]	19.25 ± 2.14[b]

a $p < 0.05$, compared to the intact group fed on the same diet (hormonal effect)
b $p < 0.05$, compared to the group fed on the control diet (dietary effect).

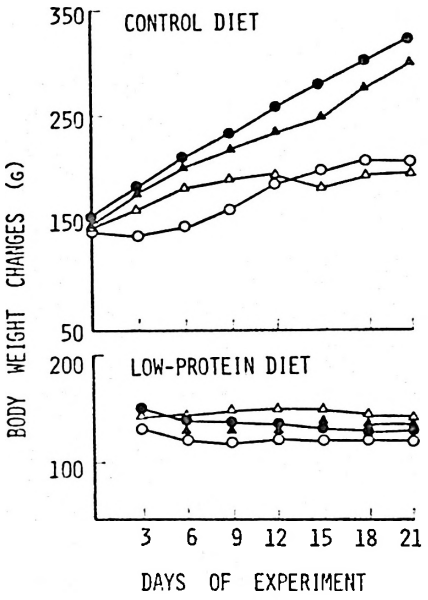

Fig. 1. *Daily body weight changes of rats receiving different insulin and dietary treatments.*
Values are the mean for five rats in each of the following hormone-treated groups: intact, receiving vehicle injection (0.9 % NaCl) (●), diabetic, receiving vehicle injection (○), diabetic receiving 0.25 I. U. of insulin/100 g body weight/day (▲), and diabetic receiving 0.50 I. U. of insulin/100 g body weight/day (△). Injections were given intraperitoneally for 21 days. Animals were fed *ad libitum* on either an adequate control diet (18 % lactalbumin) or a protein-deficient diet (1 % lactalbumin). Least significant differences (for $p < 0.02$) for the groups fed on the control and low-protein diets are 26.7 and 6.1 respectively.

weights of livers among the experimental groups. The three muscles dissected, representing predominatly red (soleus), white (extensor digitorum longus) and mixed (gastrocnemius) fibers, exhibited different responses. Relative to unit of body weight, gastrocnemius was significantly reduced ($p < 0.05$) in the diabetic animals fed on the low-protein diet. No differences were found in extensor digitorum longus muscle. However, in soleus muscle, an improved growth was recognized in all the groups fed on the low-protein diet, compared to those fed on the control diet ($p < 0.05$). Food intake, expressed as g of agar-diet/100 g body weight, resulted significantly ($p < 0.05$) increased, in the two dietary treatments, in the diabetic animals, receiving or not insulin replacement, and it was even more pronounced in the group of rats fed on the protein-deficient diet, compared to the intact rats.

Excretion of urea-N and 3-methylhistidine. The data for urea-nitrogen and for 3-methylhistidine output are presented in figures 2 and 3 respectively. Urinary urea-N excretion in rats fed on the control diet, was significantly elevated ($p < 0.02$) in the diabetic rats receiving or not hormonal treatment, as compared to the intact con-

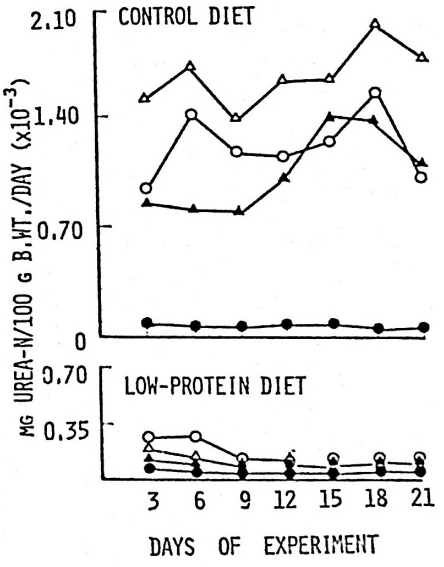

Fig. 2. *Daily output of urea-N/100 g body weight/day.*
Each point is the value from a pooled sample of five rats. Least significant differences (for $p < 0.02$) for the groups fed on the control diet and low-protein diet are 44.7 and 218.6 respectively. Explanation of the symbols and other details are as described in legend to fig. 1.

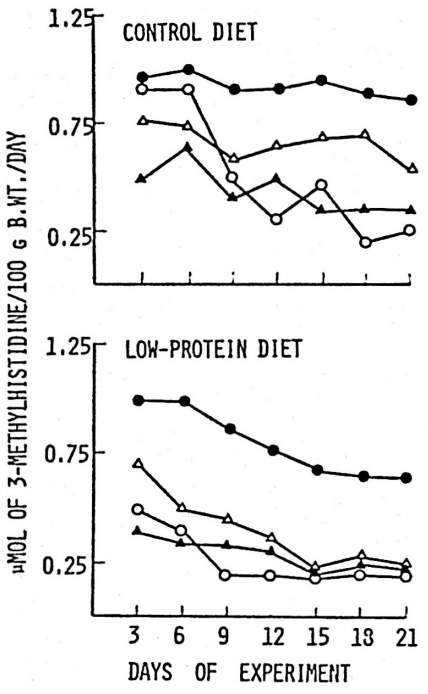

Fig. 3. *Daily output of 3-methylhistidine/100 g body weight/day.*
Each point is the value from a pooled sample of five rats. Least significant differences (for $p < 0.02$) for the groups fed on the control diet and low-protein diet are 0.24 and 0.14 respectively. Explanation of the symbols and other details are as described in legend to fig. 1.

trol. Administration of 0.25 I.U. of insulin led to a reduced output of this metabolite, as compared to the diabetic receiving vehicle injection, and to the diabetic, receiving 0.50 I.U. of insulin. Lack of protein in the diet brought all urinary urea-N values almost to the range of the intact rats fed on the control diet. Diabetic animals displayed a low level of output of 3-methylhistidine, as compared to the intact animals in both dietary treatments. This reduction was particularly evident in the protein-restricted rats, in which the levels decreased at a constant rate throughout the experiment, once a decline was evident. The diabetic groups receiving no insulin replacement, and fed on the control diet, initially, between days 3 and 5, displayed an increased output (reaching the values of the intact animals), but later, it decreased to the same range as the other groups.

Plasma hormone concentrations. The plasma insulin levels obtained on the day of killing are presented in table II. These values confirmed that rats were diabetic, as well as demonstrated the extent of the replacement provided by insulin treatment.

Discussion

The interrelationship between diet, hormonal status and growth was specially evident through the observation of body weight during the course of the experiment. As shown by these changes, diabetic animals displayed a reduced rate of growth compared to the intact animals, even on the control diet, gaining only 60 g over the three weeks of the experiment, compared to the 125 g gained by the intact animals. Both, diabetic and intact animals did not grow on the protein deficient diet (13), which means that in the present experiment, restriction of protein in the diet was a limiting factor in regarding to growth in the rats under the diabetic state. Replacement with insulin allowed the diabetic rats to gain additional weight. The elevated food intake observed by diabetic animals fed on either diet, and expressed per unit of body weight, is in agreement to the hyperfagia reported by PENG *et al.* (27), in alloxan-diabetic rats. Liver size per unit of body weight was not affected neither by the diabetic status nor the absent of protein in the diet. Concerning to the different muscles excised, it should be pointed out that only in the diabetic animals fed on the low-protein diet there was a reduction in gastrocnemius weight (mixed fibers), but no effect was noted in extensor digitorum longus muscle

(white fibers); however, soleus muscle resulted to be increased in the different groups of rats fed on the protein deficient diet. The commonly recognized catabolic effect of diabetes (7) did not bring about in this experiment a serious reduction in muscle weight. TOMAS *et al.* (31) studying the catabolic action of glucocorticoids were not able to find any effect on extensor digitorum longus muscle, as well as in soleus. This latter muscle (red fibers) is a highly active metabolic muscle (10), and as it has been postulated by HENNEMAN and OLSON (15), in the catabolic status originated by different hormones, the body mobilizes its protein from the less active muscles (the pale ones), sparing those physiologically more active, such as soleus.

The observed catabolic state is further supported by elevated levels of urea-N by the diabetic animals, receiving or not insulin replacement, and fed on the control diet. This could be due to an inefficient reutilization of the amino acids of the diet, and as indicated by several workers (12, 20), insulin decreases the rate of amino acids efflux from various tissues, and therefore, a diabetic status would lead to an increase in the rate of amino acids going to catabolic pathways, causing an increase in the total urinary nitrogen excretion, as noted by FELIG *et al.* (8) and LUNDHOLM *et al.* (19). However, animals fed on the low-protein diet exhibited a low urea-N excretion in all experimental groups, indicating a possible adaptation to the low protein intake, in order to conserve the whole body protein (13). Thus, the diabetic state is one of catabolic nature that is particularly sensitive to the protein content in the diet.

Concerning to the plasma insulin levels, ATINMO *et al.* (2) found in pigs that low-protein diets resulted in a persistent low insulin levels in plasma. Other investigators (22, 32, 37) confirmed that restrictions in the protein content in the diet would decrease plasma insulin levels in rats and humans, in which an impairment in the pancreas function was reported as a consequence of protein malnutrition. On the other hand, it should be noted that plasma insulin levels in the diabetic rats receiving the high dose of the hormone were lower than that of those receiving the 0.25 I.U. of insuline. It seems probable that, as pointed out by SACK *et al.* (30), high doses of insulin would activate the insulin-degrading enzymes system, causing an acceleration in the rate of metabolization of the hormone, which would be noted by reduced hormonal plasma levels. But, since experimentally we can only provide a short term dose, compared to the continuous and regulated excretion of the healthy pancreas (34), the experimental replacement in this experiment appeared to «take the edge» off the diabetic state.

The last objective of this paper was to assess the involvement of the *in vivo* muscle protein breakdown in the rats receiving different dietary and hormonal treatment. Diabetic animals fed on a low-protein diet showed a reduced excretion of 3-methylhistidine throughout the experiment, indicating a reduced level of muscle protein breakdown, and, again, showing that diabetes is very sensitive to the amount of protein present in the diet. On the other hand, insulin stimulates the rate of muscle protein synthesis (8, 19), and therefore, it appears clear that a reduction in the protein would imply a reduction in the rate of muscle protein breakdown, so that muscle may conserve its protein integrity (14). Besides, insulin inhibits protein breakdown in several tissues (5, 11, 16, 17, 24, 25). The results of the present experiment indicated that the rate of myofibrillar breakdown was diminished during diabetes, and this was so, specially on protein malnutrition. Our data do not agree with the proposition that during insulin deficiency there is an increase in protein catabolism. However, the possibility that the initial response to diabetes could be mediated in part by an

increase in muscle protein degradation cannot be excluded, since, experimentally, the administration of insulin began 14 days after the administration of streptozotocin. It can be possible, that by this time, the animals were already adapted to the insulin deficiency by decreasing muscle protein degradation in order to conserve their muscle mass. Besides, MILLWARD *et al.* (23) have determined the fractional rates of protein synthesis and breakdown in skeletal muscle of diabetic rats, using the constant infusion of labeled amino acids technique, finding that diabetes resulted in a reduction in the rate of protein synthesis and breakdown, in agreement to the results reported in this experiment.

Resumen

Ratas macho diabéticas (streptocozina) alimentadas *ad libitum*, con una dieta control adecuada en proteína y energía o con una dieta deficiente en proteína, pero adecuada en energía. Dentro de cada uno de estos grupos se establecen tres subgrupos: ratas a las que se les inyecta vehículo, o 0,25 ó 0,50 I.U. de insulina/100 g peso corporal/día i.p. durante 21 días. Un cuarto grupo de ratas intactas se incluye como control. Cada día se controla la orina eliminada con el fin de determinar su contenido en urea y 3-metilhistidina (3-Mehis), así como el peso de los animales y la cantidad de comida ingerida. Al final del experimento, en todos los animales se determina el nivel plasmático de insulina en sangre, así como el peso de los hígados y de los músculos gastrocnemius, soleus y tibialis.

Los resultados indican que las ratas diabéticas pesan menos que los animales intactos, aunque la cantidad de comida ingerida es elevada en todos los grupos. Los pesos de los músculos gastrocnemius y tibialis son respectivamente menores y mayores en los animales diabéticos alimentados con la dieta baja en proteína que en los animales controles. La excreción de urea aumenta en todos los grupos alimentados con la dieta control, aunque experimenta una reducción significativa en los animales alimentados con la dieta baja en proteína. La excreción urinaria de 3-Mehis es más baja en los animales diabéticos, especialmente en aquellos alimentados con la dieta deficiente en proteína, en comparación con las ratas intactas alimentadas con la dieta control. Los resultados muestran que los animales diabéticos experimentan una reducción en el nivel de degradación de las proteínas miofibrilares, especialmente evidente cuando se alimentan con una dieta baja en proteína.

Acknowledgement

This study was supported by a National Institute of Health Grant AM 16654. The assistance given by Mr. Savitt and Mrs. Morcyra, as well as Mr. Sobrini and Miss Bilmazes is acknowledged. This study was carried out during S. S.'s leave of absence for Post-Doctoral training at MIT from the Department of Physiology (Division of Nutrition), Universidad de Navarra. Pamplona, Navarra, Spain.

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