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ORIGINAL DATA

Effects of Whey Proteins on Glucose Metabolism in Normal Wistar Rats and Zucker Diabetic Fatty (ZDF) Rats

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■ Abstract

BACKGROUND: Beneficial effects of milk protein on glucose metabolism have been reported. **OBJECTIVES**: We hypothesized that dietary supplementation with specific milk protein fractions could prevent diabetes and differentially alter tissue gene expression. Therefore, we studied the effects of supplementing the diet with whey isolate, whey hydrolysate, α-lactalbumin, and casein proteins in Zucker Diabetic Fatty rats (ZDF) and normal Wistar rats. A chow diet was included as well. **METHODS**: Six week old male ZDF (n = 60) and Wistar rats (n = 44) were used in a 13 week study. P-glucose, p-insulin, p-glucagon, HbA1c, total-cholesterol, HDL-cholesterol, and triglycerides were measured. An oral glucose tolerance test (OGTT) was performed. Liver, muscle, and adipose samples were used for RT-PCR. One-way ANOVA and multiple comparison tests were performed. **RESULTS**: HbA1c increased during intervention, and was significantly lower for all milk protein fractions compared to chow in the ZDF rats ($p < 0.05$). At week 18, iAUCs during OGTT in the ZDF rats were similar for all milk protein-treated groups and significantly lower than in the chow fed group ($p < 0.01$). In the chow-fed group of ZDF rats, p-glucagon increased significantly compared to all milk protein fed animals. Total and HDL cholesterol were increased in the milk protein-treated ZDF rats compared with the control group. Expression of liver GYS2 and SREBP-2 were downregulated in the milk protein-fed ZDF groups compared with chow. **CONCLUSIONS**: We conclude that milk protein fractions improve glycemic indices in diabetic rats. No major differences were seen between the milk protein fractions. However, the fractions had a differential impact on tissue gene expression, most pronounced in ZDF rats.

Keywords: type 2 diabetes · milk protein · whey protein · casein · Zucker rat · Wistar rat · ZDF · glucose tolerance test · OGTT · gene expression

Introduction

The high prevalence and incidence of obesity and type 2 diabetes (T2D) call for new ways to reduce the risk of T2D and cardiovascular disease. Dietary means of preventing the obesity problem is of major importance. Consumption of milk and dairy products has been associated with reduced risk of diabetes [1]. Milk contains two primary sources of protein, casein and whey. Recently, the beneficial physiological effects of whey protein on the control of food intake and glucose metabolism have been reported [2]. Studies have shown insulinotropic and glucose-lowering properties of whey protein in healthy and T2D subjects [2, 3]. Diets high in whey protein and isolates have therefore been of interest in weight management and as promoters of muscle protein synthesis [4].

We have previously shown that whey protein acutely lowers postprandial triglyceride levels in obese [5] and T2D subjects ingesting a fat-rich meal [6]. Whey protein contains high amounts of branched chain amino acids, β-lactalbumin, α-lactalbumin, immunoglobulins, and albumin [7].

Abbreviations:
ACE - angiotensin-converting enzyme
ANOVA - analysis of variance
BW - body weight
cDNA - complementary DNA
CPT-1 - carnitine palmitoyltransferase 1
CRP - C-reactive protein
Ct - cycle threshold
DNA - deoxyribonucleic acid
DNase - deoxyribonuclease
DPP-4 - dipeptidylpeptidase 4
GLP-1 - glucagon-like peptide 1
GLUT-2 - glucose transporter 2
GYS2 - glycogen synthase 2
HbA1c - glycosylated hemoglobin
HDL - high-density lipoprotein
HOMA-IR - homeostasis model assessment - insulin resistance
HPRT1 - hypoxanthine phosphoribosyltransferase 1
iAUC - incremental area under the curve
IL-6 - interleukin 6
InsR - insulin receptor
IR - insulin resistance
JNK - c-Jun N-terminal kinase
OD - optical density
OGTT - oral glucose tolerance test
PGC-1α - PPAR gamma coactivator 1 alpha
PPAR - peroxisomal proliferators-activated receptor
RIA - radioimmunoassay
RNA - ribonucleic acid
RT-PCR - real-time polymerase chain reaction
SD - standard deviation
SEM - standard error of mean
SREBP-2 - sterol regulatory element-binding protein 2
T2D - type 2 diabetes
TNF-α - tumor necrosis factor alpha
ZDF - Zucker Diabetic Fatty (rat)

The potential of specific milk protein fractions to prevent T2D needs clarification. Furthermore, our knowledge is sparse on how the different protein fractions modulate tissue-specific key regulatory genes involved in glucose- and lipid-metabolism as well as inflammation.

We hypothesized that enrichment of the diet with various milk protein fractions can postpone the progression of diabetes. To study this hypothesis we compared the effects of whey isolate, whey hydrolysate, α-lactalbumin, and casein and also included a standard chow diet in Zucker Diabetic Fatty (ZDF) and normal Wistar rats in a 13-week intervention study.

Materials and methods

Design

In the diet intervention study, we used 6 week old animals, 60 male ZDF rats and 44 male Wistar rats (control). ZDF rats were used for the study of T2D; they have a leptin receptor mutation [8]. Before intervention, rats were fed a standard chow diet (Altromin 1324, Altromin GmbH, Lage, Germany). The two rat strains were each divided into five intervention groups (n = 12/group for ZDF rats, n = 8-9/group for Wistar rats):

1. Whey protein isolate
2. Whey protein hydrolysate
3. α-lactalbumin
4. Casein
5. Standard chow (1324, Altromin, Lage, Germany)

The animals were housed in individual cages at 21°C, with a 12-hour light:dark cycle. The experiment was performed in accordance with the Danish Animal Ethics Council.

Blood sampling was conducted before and at the end of the experiments, and blood glucose, body weight, and 24-hour food intake were measured every fortnight.

During the last week of the intervention an oral glucose tolerance test (OGTT) was performed. At the end of the intervention, animals were sacrificed, and blood and tissue samples were taken for hormone and plasma lipid examinations and gene expression studies.

Diets

The whey and casein protein fractions were provided by Arla Foods Ingredients Group P/S (Viby J, Denmark) and incorporated into pellets by Altromin (Lage, Germany). We applied the following three different whey protein fractions:

1. Whey protein isolate
2. Whey protein hydrolysate
3. α-lactalbumin

Hereafter, the fractions are referred to as isolate, hydrolysate, and α-lactalbumin.

The macronutrient composition of the diets is shown in **Table 1**. A macronutrient analysis was performed by the Danish Veterinary and Food Administration, Region Nord. The relative composition of macronutrients and energy densities were close to similar in the protein groups, whereas the chow group had higher carbohydrate and lower protein content as intended. The standard chow was 1324 from Altromin containing 19% crude protein.

Table 1. Macronutrient composition (unit/100g) of the five experimental diets used in the intervention study

Component/ energy	Whey protein			Casein	Chow
	Isolate	Hydrolys.	α-lactalb.		
Carbohydrate (g)	41.1	45.1	42.2	41.2	53.0
Protein (g)	48.6	42.4	49.1	49.0	19.0
Fat (g)	5.1	5.1	6.1	5.8	4.0
Energy (kJ)	1680	1646	1739	1709	1344

Blood sampling

Blood samples were collected from the tip of the tail at week 6. Chilled tubes containing a 3 μl mix of aprotinin/heparin (7.7 mg/ml / 2.300 IU/ml) were used. Samples were immediately centrifuged (4.000 rpm, 10 minutes, 4°C), and plasma was collected and frozen for later analysis of plasma (p)-glucose, p-insulin, p-glucagon, total-cholesterol, HDL-cholesterol, and triglycerides. Whole blood was collected separately in ethylenediaminetetraacetic acid-preserved collection tubes for hemoglobin A1c (HbA1c) analysis.

Oral glucose tolerance test

At week 18, an OGTT was performed after a 12-hour fast. D-glucose was administered via an oral gavage tube (2 g/kg body weight (BW)). Whole blood sampled from the tail was analyzed on a glucose meter (OneTouch Precision XCeed, Abbott Laboratories A/S, Denmark) at time points -15, 0, 30, 60, 90, 120, and 180 minutes. The trapezoidal method was applied for calculation of the incremental area under the curve (iAUC) for glucose measurements obtained at the OGTT [9].

HOMA-IR

As a surrogate measure of insulin sensitivity we applied the HOMA-IR model using the equation HOMA-IR = fasting p-glucose (mM) x fasting p-insulin (mU/l) / 22.5. The equation is considered to be a predictor of total body insulin sensitivity also in rats [10].

Tissue sampling

At the end of the intervention study (week 19) the animals were anaesthetized using pentobarbital (50 mg/kg BW), and blood sampling was performed in the animals via the intra-orbital plexus for (same samples as week 6). After cervical dislocation a midline laparotomy was performed in order to obtain liver and adipose samples. A muscle sample was taken from the soleus muscle on both hind legs. All tissue samples were frozen immediately in liquid nitrogen for subsequent gene expression studies.

Gene expression analyses

Real-time (RT) polymerase chain reaction (PCR) was performed on liver, adipose and muscle tissue.

Tissue samples (20 mg liver, 100 mg fat, 30 mg muscle) were homogenized using tissue specific Qiagen RNeasy Kits on a Qiacube (Qiagen, Copenhagen, Denmark) according to manufacturer's instructions and RNA purification using RNeasy spin columns. Traces of genomic DNA were removed by DNase treatment. Quality control was performed using optical density (OD) measurements and gel electrophoresis.

Hereafter, reverse transcription was carried out using iScript™ cDNA synthesis kit (Biorad, Hercules, CA, USA). The RT-PCR was carried out using the TaqMan assays consisting of pre-designed probes and primers. 18S and Hprt1 were used as reference genes. Aros Applied Biotechnology A/S (Skejby, Denmark) performed the RT-PCR on the liver, muscle and adipose tissue using Fluidigm's Biomark™ real-time PCR system (San Francisco, California, USA). A total of 30 specific genes and two reference genes were analyzed in triplicate.

Efficiencies between 80% and 100% and correlation coefficients above 0.995 were accepted. A variation of 5% between the analyses of the same gene was accepted. The relative gene expression was calculated using the $2^{-\Delta Ct}$ method. This method calculates the relative abundance of each gene based on the average cycle threshold number (Ct) of the triplicates and the geometric mean of the Ct values of the two reference genes (18S and HPRT1).

Analyses

At week 6 and 19, plasma concentrations of glucose, hormones and lipids were determined as follows. Insulin and glucagon were measured using RIA kits for rat insulin and glucagon (Linco Research Inc., St Charles, MO, USA). P-glucose, total cholesterol, HDL-cholesterol, triglyceride, and HbA1c were all analyzed using enzymatic colorimetric methods on a COBAS c111 (Roche Diagnostics Intl. Ltd., Switzerland).

Table 2. Food intake (g) measured during a 24-hour period every second week in the 13 week intervention with milk protein fractions in ZDF and Wistar rats, respectively

Model	Group	Week					
		7	9	11	13	15	17
ZDF rat	Isolate	21.8 ± 4.3	18.9 ± 2.3[g]	20.1 ± 1.7[e]	25.7 ± 4.7[e]	23.7 ± 2.5[d]	20.3 ± 6.3[d]
	Hydrolysate	22.2 ± 4.2	20.6 ± 4.9[f]	20.3 ± 7.0[f]	25.1 ± 4.9[f]	25.2 ± 4.4[d,i]	20.2 ± 2.7[d]
	α-lactalbumin	20.7 ± 4.0[a]	19.4 ± 2.6[g]	20.5 ± 2.5[e]	25.0 ± 3.0[e]	24.4 ± 2.9[f,h]	19.9 ± 5.6[d]
	Casein	19.9 ± 2.9[b]	18.7 ± 1.4[g]	20.4 ± 2.5[e]	21.5 ± 2.1[g]	19.6 ± 2.0[f]	16.2 ± 2.5[d,h]
	Chow	25.9 ± 3.8	29.4 ± 3.3	27.4 ± 3.3	34.6 ± 2.6	31.9 ± 3.5	33.5 ± 3.6
Wistar rat	Isolate	19.7 ± 1.8[d]	18.7 ± 2.0[c]	17.3 ± 2.9[d]	16.4 ± 4.1[g]	18.5 ± 2.0[a]	18.0 ± 1.9[a]
	Hydrolysate	21.5 ± 3.2[c,j]	20.5 ± 3.8[b]	18.9 ± 2.7[c]	19.5 ± 2.8	18.2 ± 1.9[a]	19.4 ± 2.7
	α-lactalbumin	17.4 ± 2.1[d]	18.6 ± 2.4[d]	17.6 ± 2.6[d]	17.3 ± 1.7[f]	16.9 ± 2.4[b]	17.5 ± 2.1[b]
	Casein	18.2 ± 1.6[d]	19.5 ± 3.3[c]	19.7 ± 3.6[c]	18.2 ± 1.4[e]	18.3 ± 2.5[a]	18.7 ± 2.4
	Chow	27.4 ± 3.2	28.5 ± 5.6	26.6 ± 3.5	25.0 ± 1.9	22.9 ± 4.7	22.4 ± 3.7

Legend: Data are means ± SEM, n(ZDF) = 12/group, n(Wistar) = 8-9/group. [a] $p < 0.05$, [b] $p < 0.01$, [c] $p < 0.001$, [d] $p < 0.0001$ vs. chow (one-way ANOVA). [e] $p < 0.05$, [f] $p < 0.01$, [g] $p < 0.001$ vs. chow (Kruskal-Wallis nonparametric test). [h] $p < 0.05$, [i] $p < 0.01$ vs. casein (one-way ANOVA). [j] $p < 0.05$ vs. α-lactalbumin (one-way ANOVA).

Statistics

Statistical tests were performed using GraphPad Prism version 5.0 (GraphPad Software Inc., La Jolla, CA, USA). Data were expressed as mean ± SEM, unless stated otherwise. Homogeneity of variances was calculated using Bartlett's test. One-way analysis of variance (ANOVA) was performed to test overall group differences. Hereafter, the multiple comparison test Bonferroni was performed. In cases where the data were not normally distributed, data were logarithmically transformed. If the assumptions were still not met, the nonparametric Kruskal-Wallis test followed by Dunn's multiple comparisons test was applied. $P < 0.05$ was considered significant.

Results

Food intake and body weight

Twenty-four hour food intake was measured every second week from start to week 17. With the exception of a lower daily food intake for the casein treated group of ZDF rats, the food intake was similar in the other high-protein fed groups. At all points in time, in both ZDF and Wistar rats the chow fed groups had a higher food intake than the high protein fed groups (**Table 2**).

Body weight from start to week 18 is shown in **Table 3**. For both ZDF and Wistar rats no significant differences were observed at week 18.

Plasma and whole blood glucose

The fasting whole blood glucose was measured every second week from the tip of the tail using a portable glucose meter. As can be seen from **Table 4**, there was a gradual increase in the fasting blood glucose for all groups of ZDF animals as expected. The rise in blood glucose was most pronounced for the chow-fed group animals. Overall, the dietary interventions in the Wistar rats did not result in changes in the blood glucose levels.

P-glucose was also measured at the beginning and at the end of the intervention. No significant differences between groups were detected when comparing changes in p-glucose (**Figure 1**).

HbA1c

HbA1c was determined at the start and the end of the intervention. The HbA1c increases (above baseline) are shown in **Figure 2**. For the diabetic rats the chow group showed significantly larger increases during the intervention compared to all other milk protein groups (**Figure 2A**). The casein groups showed the lowest increase in HbA1c compared to all other groups. For the Wistar rats no changes were seen between intervention groups (**Figure 2B**).

OGTT

One week before termination an OGTT was performed. As shown in **Figure 3A** for the ZDF

Table 3. Body weight (g) measured weekly during the 13 week intervention with milk protein fractions in ZDF and Wistar rats, respectively

Model	Group	Week						
		6	8	10	12	14	16	18
ZDF rat	Isolate	203.0 ± 5.0	308.0 ± 11.0	370.0 ± 11.0	431.0 ± 11.0	468.0 ± 10.0	498.0 ± 10.0	528.0 ± 10.0
	Hydrolysate	200.0 ± 6.0	296.0 ± 13.0	365.0 ± 13.0	417.0 ± 13.0	458.0 ± 13.0	493.0 ± 13.0	493.0 ± 13.0
	α-lactalbumin	206.0 ± 6.0	298.0 ± 6.0	333.0 ± 14.0	408.0 ± 6.0	454.0 ± 5.0	490.0 ± 6.0	524.0 ± 9.0
	Casein	210.0 ± 6.0	300.0 ± 13.0	344.0 ± 13.0	398.0 ± 15.0	426.0 ± 16.0	447.0 ± 15.0	475.0 ± 17.0
	Chow	206.0 ± 5.0	279.0 ± 5.0	343.0 ± 6.0	392.0 ± 7.0	425.0 ± 9.0	455.0 ± 11.0	485.0 ± 14.0
Wistar rat	Isolate	195.0 ± 5.0	272.0 ± 7.0	318.0 ± 8.0	347.0 ± 10.0	365.0 ± 11.0	387.0 ± 12.0	404.0 ± 12.0
	Hydrolysate	198.0 ± 4.0	270.0 ± 7.0	304.0 ± 14.0	351.0 ± 6.0	378.0 ± 7.0	404.0 ± 8.0	423.0 ± 8.0
	α-lactalbumin	193.0 ± 7.0	263.0 ± 7.0	315.0 ± 9.0	349.0 ± 8.0	382.0 ± 7.0	407.0 ± 9.0	426.0 ± 8.0
	Casein	198.0 ± 6.0	278.0 ± 3.0	324.0 ± 3.0	348.0 ± 4.0	366.0 ± 5.0	380.0 ± 6.0	392.0 ± 7.0
	Chow	183.0 ± 6.0	275.0 ± 9.0	312.0 ± 10.0	338.0 ± 12.0	355.0 ± 12.0	392.0 ± 13.0	410.0 ± 11.0

Legend: Data are means ± SEM, n(ZDF) = 12/group, n(Wistar) = 8-9/group.

rats the incremental area under the curve (iAUC) was similar for all high protein treated groups. The chow fed group had larger iAUC compared to all high protein diets (**Figure 3A**). No significant differences were observed between iAUCs for the Wistar rats (**Figure 3B**).

Plasma insulin and glucagon

P-insulin and p-glucagon were measured at the beginning and at the end of the intervention. **Figures 4** and **5** show p-insulin and glucagon levels after and before the interventions. For both ZDF and Wistar rats no significant changes for p-insulin were induced by either diet type (**Figures 4A** and **4B**). The change in p-glucagon in the ZDF rats was similar between the protein treated groups and significantly lower than for the chow fed (**Figure 5A**) whereas no changes could be detected for p-glucagon in the Wistar rats (**Figure 5B**).

HOMA-IR

To assess the impact of dairy proteins on insulin resistance HOMA-IR was calculated for both rat types after the intervention. As expected, the HOMA-IR was higher for the ZDF rats (mean 163 ± 10) than for the Wistar rats (mean 30 ± 4). No significant differences between intervention groups were found for HOMA-IR (data not shown).

Plasma lipids

Circulating levels of total cholesterol, HDL-cholesterol and triglycerides were determined at the beginning and at the end of the intervention. For the ZDF rats, total cholesterol levels increased significantly more in the three whey protein fed groups compared to both casein and chow (**Figure 6A**). Casein and chow did not differ significantly. HDL cholesterol levels seemed to increase in all the protein fed groups compared to chow, but only isolate, hydrolysate and casein showed a statistically significant increase compared to chow (**Figure 6C**). Triglyceride levels increased in all groups. The rise in triglycerides was remarkably higher in the whey fraction groups and chow compared to casein, but only hydrolysate and α-lactalbumin were statistically significantly increased compared to casein (**Figure 6E**).

For normal rats, total cholesterol levels slightly decreased during the intervention with no statistically significant differences between groups (**Figure 6B**). With the exception of the casein group, a small decrease in HDL cholesterol was noticed for all other groups. The decrease in HDL cholesterol was significant in the α-lactalbumin and chow fed groups compared with the casein group. The decrease in the chow group was also statistically larger compared to whey hydrolysate (**Figure 6D**). No significant changes in triglyceride levels were found (**Figure 6F**).

Gene expression

The expression levels of selected genes primarily involved in glucose and lipid metabolism as well as inflammation were studied after the intervention in both ZDF and Wistar rats in liver, muscle and fat tissue, respectively (**Tables A1-A6**, in

Table 4. Fasting blood glucose (mmol/l) taken as tail blood during the dietary intervention with milk protein fractions in ZDF and Wistar rats, respectively

Model	Group	Week					
		8	10	12	14	16	18
ZDF rat	Isolate	4.8 ± 0.1	4.8 ± 0.2	6.0 ± 0.3	6.1 ± 0.3	6.1 ± 0.3	6.6 ± 0.3
	Hydrolysate	4.1 ± 0.2	4.7 ± 0.2	5.1 ± 0.2	5.1 ± 0.3	5.4 ± 0.2	5.6 ± 0.2
	α-lactalbumin	4.7 ± 0.2[a]	5.6 ± 0.4	5.8 ± 0.5[a]	6.4 ± 0.4	6.9 ± 0.5	7.4 ± 0.9
	Casein	5.1 ± 0.1	4.5 ± 0.2	5.9 ± 0.3	5.1 ± 0.1	5.8 ± 0.4	5.7 ± 0.2
	Chow	4.4 ± 0.2	4.4 ± 0.1	5.1 ± 0.3	5.8 ± 0.5	7.0 ± 1.0	8.1 ± 1.0
Wistar rat	Isolate	4.9 ± 0.3[b]	4.3 ± 0.2	3.9 ± 0.2	4.0 ± 0.1	4.2 ± 0.2	4.2 ± 0.2
	Hydrolysate	4.3 ± 0.1[c]	4.6 ± 0.3	4.2 ± 0.1	4.0 ± 0.1	4.1 ± 0.1	4.1 ± 0.2
	α-lactalbumin	4.0 ± 0.3	4.5 ± 0.2[a]	4.6 ± 0.2	4.4 ± 0.4[d]	4.1 ± 0.1	4.4 ± 0.2
	Casein	4.3 ± 0.2	3.8 ± 0.3	3.7 ± 0.2	3.9 ± 0.3	3.6 ± 0.3	3.8 ± 0.2
	Chow	5.2 ± 0.2	3.8 ± 0.1	4.1 ± 0.2	4.0 ± 0.2	4.0 ± 0.2	4.3 ± 0.2

Legend: Data are means ± SEM, n(ZDF) = 12/group, n(Wistar) = 8-9/group. [a] $p < 0.05$ vs. chow (one-way ANOVA). [b] $p < 0.05$ vs. hydrolysate (one-way ANOVA). [c] $p < 0.01$ vs. chow (one-way ANOVA). [d] $p < 0.05$ vs. casein (Kruskal-Wallis nonparametric test).

the Appendix). Several gene expression changes were observed in all tissues. Fewest gene changes were found in muscle tissue. Compared to chow there was a tendency towards lower InsR and GLUT-2 expression levels. This was, however, only significant for the α-lactalbumin group for InsR and the casein group for GLUT-2. Because of low expression of TNF-α in muscle and liver tissue in ZDF and Wistar rats, and PGC-1α in liver in ZDF rats, these genes are not shown in **Tables A1-A6** (in the Appendix).

In general, few genes in Wistar rats showed significant changes. Several gene changes were however seen in the adipose tissue of Wistar rats (**Table A6**). The expression level of GLUT-4 in adipose tissue in the α-lactalbumin and casein group was higher compared with the chow group. In addition, CPT-1 expression levels were higher in the α-lactalbumin and casein groups compared to the chow group. Expression levels of FAS were higher expressed in the casein group also compared with the chow group.

Discussion and conclusions

Our study performed in both prediabetic ZDF and normal Wistar rats showed differential effects on metabolism and gene expression levels of the milk protein fractions studied. The main focus of our study was to clarify if particular milk protein fractions had the potential to affect the development and progression of diabetes in a rat model. In parallel, we compared our results in ZDF rats with those obtained in normal Wistar rats to dissect potential effects not influenced by the metabolic disturbances seen in diabetes.

In this study, the main finding was that HbA1c was lower and the glucose tolerance was increased after addition of all milk protein types given to ZDF rats, especially in the casein group. We also compared the impact of the milk protein with that of a standard chow diet. Considerable evidence indicates that milk proteins are beneficial for human health [1]. The mechanisms of action of milk protein fractions may be related to e.g. changes in circulating amino acid levels, ACE inhibition [11] and/or DPP-4 inhibition [12].

None of the milk protein fractions had long-term influences on glycemic control in our non-diabetic rats, as estimated by the lack of effects on HbA1c. In contrast, the HbA1c change (i.e. the difference between level after to level before milk protein supplementation) was significantly lower for all milk protein fractions compared with chow in the ZDF rats. Surprisingly, it occurs that the the casein fraction was most potent in this respect. This was unexpected since several previous studies have shown beneficial effects of whey protein compared to casein or other protein types [3, 13-15]. The reason for this could be the substantially lower body weight in the casein treated groups. However, this difference was not statistically significant.

In view of the relatively short intervention period, the OGTT results may provide a more reliable measure of the current glycemic state than HbA1c. Thus, the rats may not have had overt diabetes until a few weeks prior to the study end.

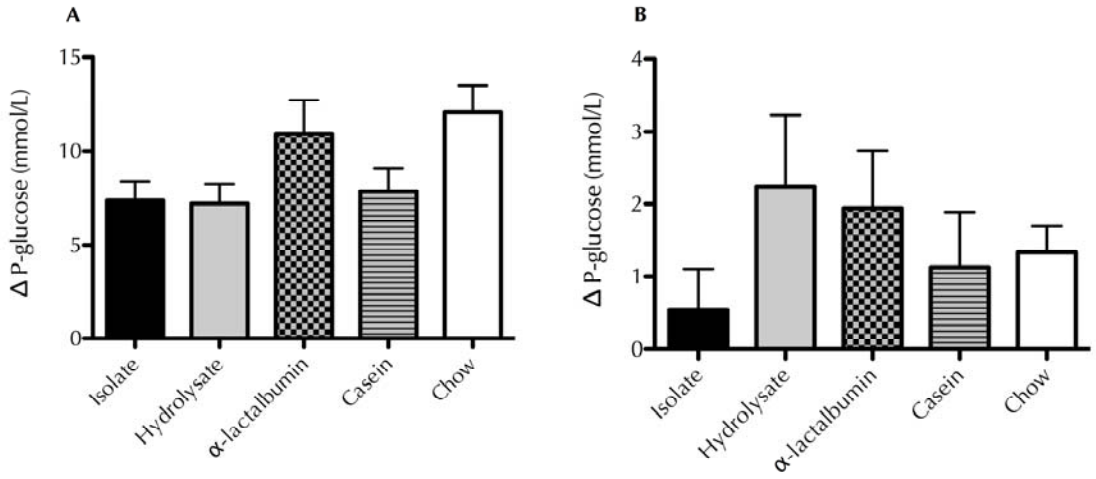

Figure 1. Changes in p-glucose. P-glucose (mmol/l) measured at baseline and after 13 weeks of intervention with four different milk protein fractions (whey isolate, whey hydrolysate, α-lactalbumin, and casein) and chow in ZDF (**A**) and Wistar (**B**) rats. The change from baseline to end is calculated and shown in percentage. Data are shown as means ± SEM, n(ZDF) = 12/group and n(Wistar) = 8-9/group.

Furthermore, all milk protein groups had significantly lower iAUC after an OGTT than the control group. Although the change in whole blood glucose was not significant, the trend towards an improvement in glycemia supports the HbA1c results. The Wistar rats had similar OGTT responses in all groups. In ZDF rats, the OGTT results seemed to correlate well with the HbA1c results. However, the casein group did not have lower iAUC during OGTT, which may reflect a similar glucose tolerance as the whey protein groups, despite differences in HbA1c. The reason for this apparent discrepancy is not clear.

The results of the HbA1c and OGTT indicate that the progression of diabetes in the whey and casein treated groups is delayed compared with the ZDF control group. Our results corroborate previous studies in high-fat fed mice where a whey protein diet was able to slow diabetes progression [16].

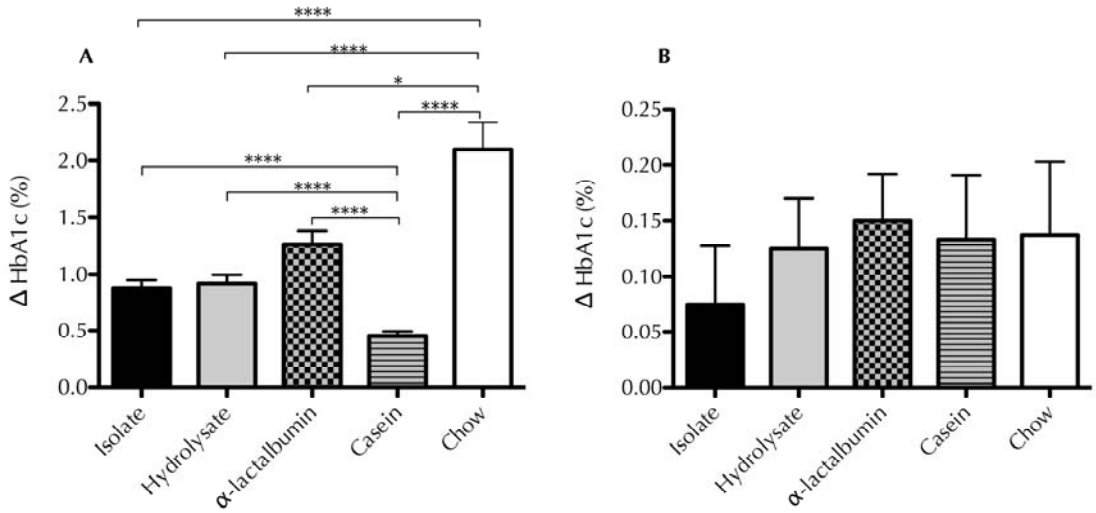

Figure 2. Changes in hemoglobin A1c. Effects on HbA1c of 13 weeks dietary intervention with four different milk protein fractions (whey isolate, whey hydrolysate, α-lactalbumin, and casein) and chow in ZDF (**A**) and Wistar (**B**) rats. Data are shown as means ± SEM, n(ZDF) = 12/group and n(Wistar) = 8-9/group. * $p < 0.05$ and **** $p < 0.0001$ (one-way ANOVA).

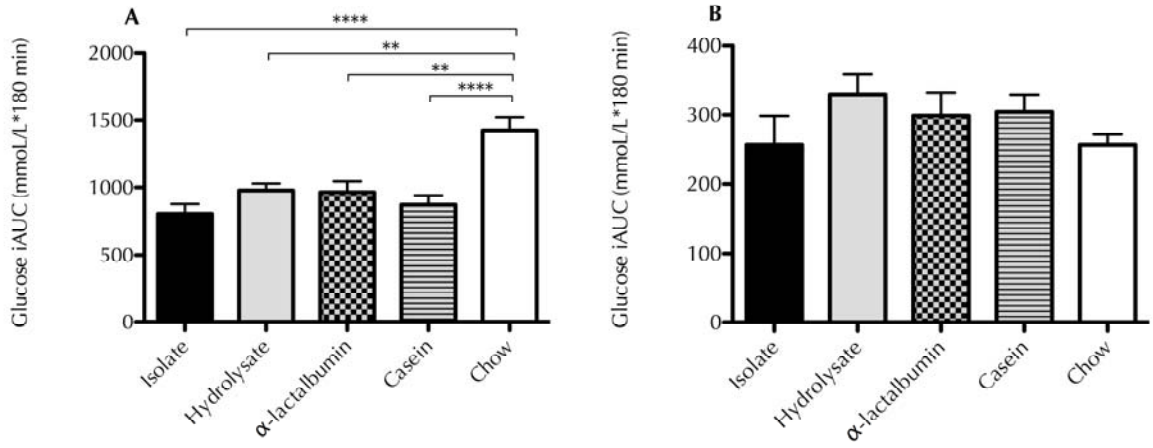

Figure 3. Evaluation of oral glucose tolerance test responses after 13 weeks of intervention. Effects on incremental areas under the curve (iAUC) for p-glucose measured by an OGTT after 13 weeks of dietary intervention with four different milk protein fractions (whey isolate, whey hydrolysate, α-lactalbumin, and casein) and chow in ZDF (**A**) and Wistar (**B**) rats. Data are shown as means ± SEM, n(ZDF) = 12/group and n(Wistar) = 8-9/group. ** $p < 0.01$ and **** $p < 0.0001$ (one-way ANOVA).

Surprisingly, neither p-insulin nor HOMA-IR were significantly changed by milk protein fractions in ZDF and Wistar rats. However, other long-term studies have shown that whey intake compared to other protein sources increases insulin sensitivity in humans [17]. Similarly, whey protein compared with no protein had beneficial effects on insulin sensitivity in mice [16]. Our HOMA-IR measurements did not confirm this. However, the HbA1c and OGTT data both indicated an improvement of glycemia in ZDF rats receiving milk proteins.

We detected significantly lower p-glucagon levels in all four milk protein groups compared with the ZDF control group. A similar pattern, although not significant, was found in normal rats. It can be speculated that milk proteins have a beneficial effect on p-glucagon levels, leading to reduced glycemia secondary to increased glucagon like peptide 1 (GLP-1), either stimulated via amino acids *per se*

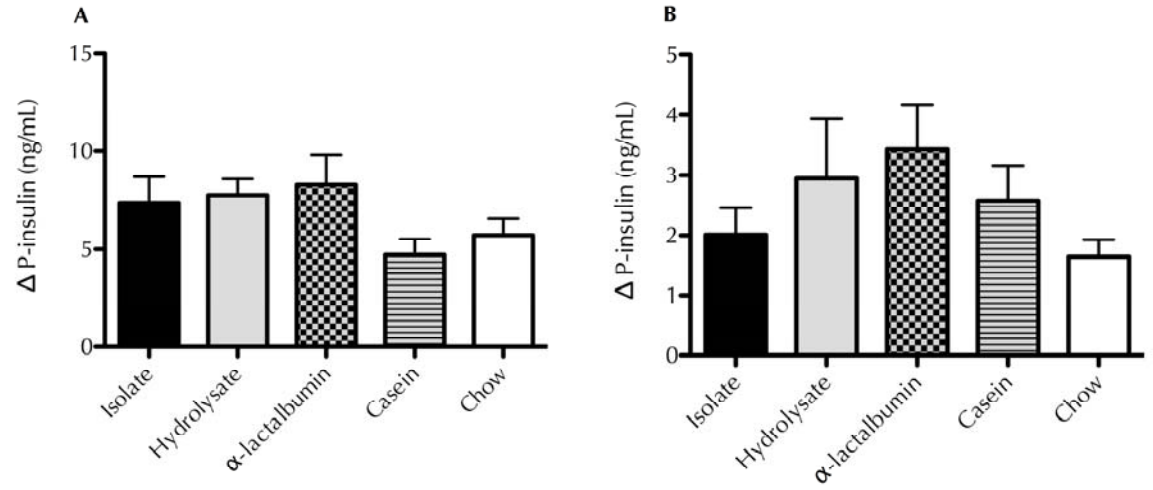

Figure 4. Changes in p-insulin. ZDF (**A**) and Wistar (**B**) rats after 13 weeks of dietary intervention with four different milk protein fractions (whey isolate, whey hydrolysate, α-lactalbumin, and casein) and chow. Data are shown as means ± SEM, n(ZDF) = 12/group and n(Wistar) = 8-9/group.

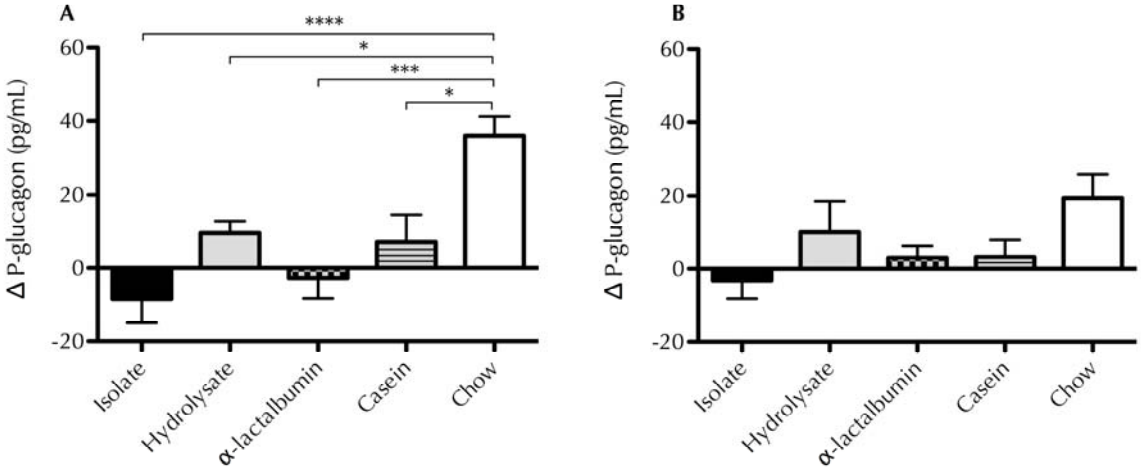

Figure 5. Changes in p-glucagon. ZDF (**A**) and Wistar (**B**) rats after 13 weeks of dietary intervention with four different milk protein fractions (whey isolate, whey hydrolysate, α-lactalbumin, and casein) and chow. Data are shown as means ± SEM, n(ZDF) = 12/group and n(Wistar) = 8-9/group. * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$. **** $p < 0.0001$ (one-way ANOVA).

or via inhibition of dipeptidyl peptidase-4 (DPP-4). In the present study, we have not measured changes in circulating amino acids, GLP-1, or DPP-4 inhibition.

Both fasting insulin and glucose levels were profoundly elevated in all ZDF groups by week 13 of the intervention. This indicated the development of severe insulin resistance in muscle and adipose tissue as well as reduced liver glycogen synthesis and storage. In the present study, only fasting insulin levels were measured. It would however also be of interest to measure postprandial insulin levels since increased postprandial insulin levels in response to whey protein have been reported in healthy and T2D subjects [14, 18, 19]. Although similar fasting insulin levels were found in all groups, it cannot be ruled out that stimulated insulin responses may have differed between groups.

When analyzing the metabolic data, one needs to consider food intake and body weight. It is evident that for both ZDF and Wistar rats the food intake was much higher in the control group of rats during the entire intervention. The food intake in the leptin-deficient ZDF rats was, as expected, higher than for normal rats, and the ZDF rats had a higher weight at the end of the intervention period. In contrast, changes in body weight were not seen in Wistar rats. ZDF rats showed different developments in body weight, with the casein and control group showing the lowest body weight rise at the end of the study, despite a higher food intake for the control group. Most likely, the control group had more severe glucosuria and loss of energy than the milk protein groups. However, this cannot account for the lower body weight in the casein-treated group. It can instead be explained by a significantly lower food intake in the casein group, which may be due to higher satiety caused by coagulated casein in the stomach.

Whether casein induced a lower food intake via increased satiety or thermogenic effect is speculative, especially in ZDF rats. In this context, it has previously been shown that a casein-based diet in rats resulted in lower weight gain compared with a whey diet, despite absence of differences in food intake. This has been explained by the lower nutritional quality of casein protein vs. whey protein [20, 21]. However, it is noteworthy that differences in protein quality did not affect weight gain in normal Wistar rats. Therefore, it is unlikely that differences in weight gain in ZDF rats can be ascribed to differences in protein quality. The energy density in the chow diet was lower (1344 kJ/100 g) than in the protein-enriched groups (1646-1739 kJ/100 g). It cannot be excluded that protein overload in the milk protein groups may had an effect on the results obtained in this study, especially when comparing with the chow group. The study was not designed to test this aspect.

Taken together, our results indicate that all studied milk protein fractions exert beneficial effects in glucose metabolism, with casein mediating

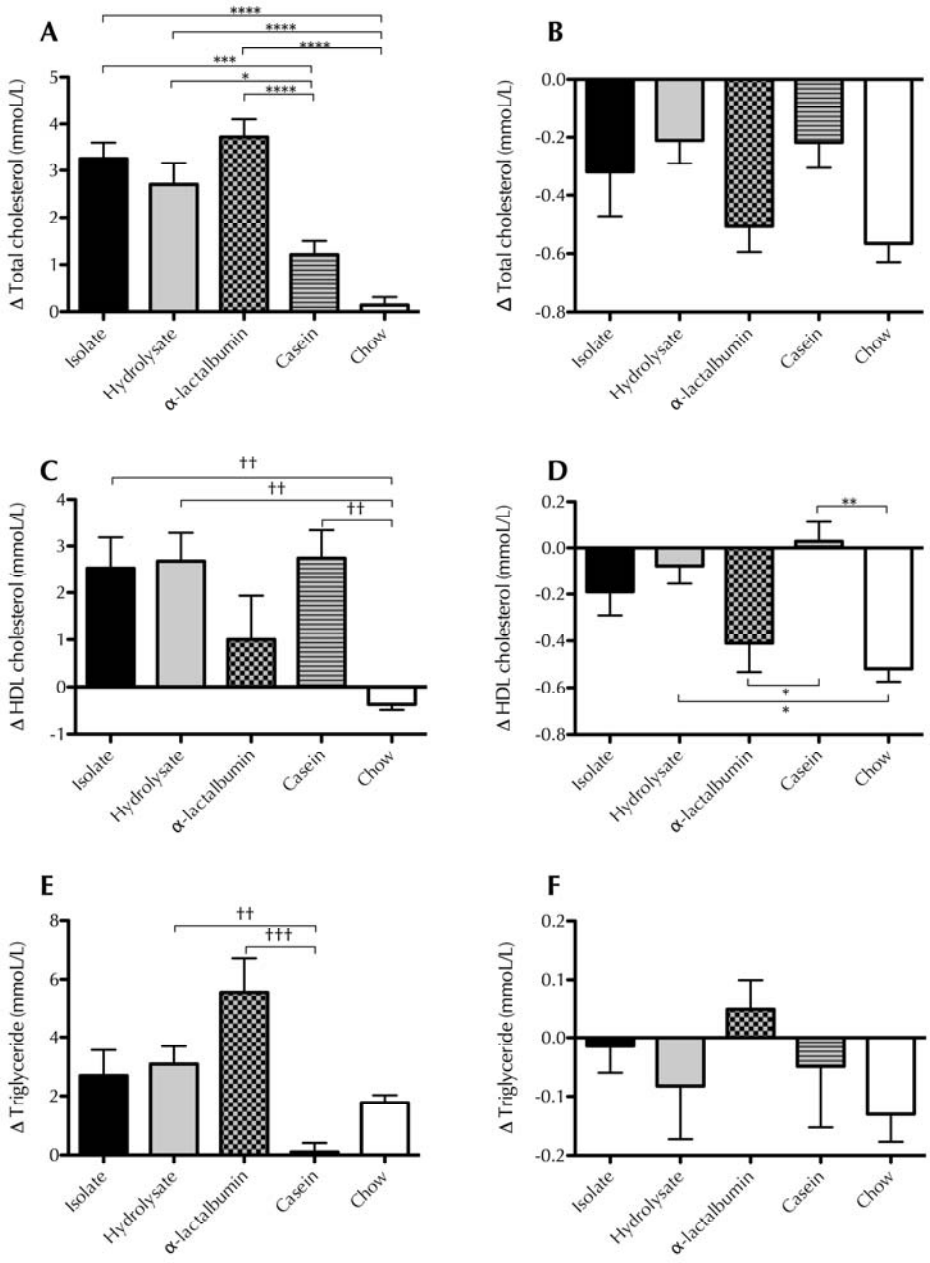

Figure 6. Change in lipid profile after 13 weeks of intervention. Effect of 13 weeks dietary intervention with whey isolate, whey hydrolysate, α-lactalbumin, casein, and chow on changes in total cholesterol, HDL-cholesterol, and triglycerides in ZDF (**A**, **C**, **E**) and Wistar (**B**, **D**, **F**) rats. Data are shown as means ± SEM, n(ZDF) = 12/group and n(Wistar) = 8-9/group. * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$. **** $p < 0.0001$ (one-way ANOVA). †† $p < 0.01$. ††† $p < 0.001$ (Kruskal-Wallis nonparametric test).

lowered HbA1c and improved glucose tolerance. In diabetic animals, improved glucose metabolism has probably been mediated by reduced food intake and lowered p-glucagon.

We found an increase in total cholesterol and HDL cholesterol in the milk protein-treated ZDF rats compared with control. Due to great variations in HDL and triglyceride data we were unable to carry out specific statistical analysis. However, it is noteworthy that casein seemed to suppress triglyceride levels. Increased HDL cholesterol and lowered triglycerides are considered to be benefi-

cial for cardiovascular risk. No major effects on lipid levels were found in the normal Wistar rats. However, HDL cholesterol was lower in the control and α-lactalbumin groups than in the casein group.

We measured gene expression changes in liver, fat, and muscle tissues to identify potential actions of the dietary milk proteins on tissue-specific key regulatory genes involved in insulin sensitivity, inflammation, glucose and fat metabolism, and transcription factors. It is noteworthy that few gene expression changes took place in normal rats, especially in liver and muscle tissue. On the other hand, there were considerably more gene expression changes in the ZDF rats. It is not known whether the observed gene expression changes are the cause or the result of alterations in glycemia and/or the observed changes in circulating lipid levels or other substances.

The finding that liver GYS2 levels were reduced in the milk protein-fed ZDF rats is consistent with the lower circulating glucose levels, presumably resulting in lower liver glycogen synthesis [22]. Furthermore, one may speculate that the net result of lowered GYS2 and lower glucagon levels can lead to reduced hepatic glucose production. Hyperglucagonemia seems to play a major role in hyperglycemia in T2D, as demonstrated by Unger *et al*. [23]. All groups had similar levels of circulating insulin. Under normal conditions, insulin suppresses glucagon secretion once released from the islet β-cells. It is likely that the α-cells have become insensitive to the inhibitory effects of insulin as a result of the diabetic state [24].

In relation to genes involved in inflammation, we studied liver CRP, IL-6, and TNF-α gene expression. In liver, CRP gene expression was significantly lower in the casein group than in the control group, whereas both the isolate and hydrolysate induced higher CRP gene expression levels. No changes were seen in the inflammatory genes in either muscle or fat tissue. As expected, the highest levels of TNF-α were found in fat tissue. No differences were seen between groups. IL-6 also showed low expression levels without group differences. TNF-α and IL-6 can cause insulin resistance by activation of JNK1, and thus interrupting insulin signaling [25]. Therefore, it is surprising that the lowest level of JNK1 expression in adipose tissue was seen in the control group; other studies have found a consistent association between JNK1 and insulin resistance [26]. As mentioned previously, we expected the ZDF control group to be most insulin-resistant. It is possible that the low level of JNK1 gene expression in the control group is a response variable not solely depending on the inflammatory status.

Adiponectin levels were shown to be inversely correlated with insulin resistance [27]. The present study found a higher adiponectin expression level in the adipose tissue of ZDF animals from the casein group. The findings that the casein- and control-fed groups had comparable body weights, and that a higher adiponectin level was seen in the casein group, pointed to increased insulin sensitivity. Earlier studies have shown that whey protein-, casein-, or high carbohydrate-rich diet have no effect on the circulating levels of adiponectin in obese subjects [28].

We found lower gene expression levels of CRP in the liver of casein-treated ZDF rats. This was in accordance with the finding of lower expression of adiponectin in the casein group. Casein seems to lower inflammation in liver and fat tissue, which may be related to an improved diabetes status. However, it is not possible to distinguish the impact of the lower weight in the casein group from that of "direct" beneficial effects of casein on metabolism and inflammation.

Several transcription factors were differentially expressed. Again, casein seemed to induce the most potent changes, at least in fat expression of e.g. the four important transcription factors PPAR-α, PPAR-γ, JNK, and SREBP-1C. In muscle, expression levels of transcription factors PPAR-γ and SREBP-1C were also increased in most milk protein-fed groups.

One may speculate that milk protein differentially changes gene expression levels towards a more beneficial metabolism in the milk protein groups compared with control. This seems to be most prominent in fat tissue. On the other hand, one may have expected that the metabolically more active tissues, i.e. liver and muscle tissue, would have been more sensitive to changes in circulating amino acid levels than adipose tissue. Therefore, it can be hypothesized that gene expression changes probably induced by milk proteins are particularly important for the fat tissue and may be involved in the redistribution or loss of adipose tissue.

Whey protein seems to induce its beneficial effects via amino acids generated during its gastrointestinal digestion. These amino acids and peptides stimulate the release of several gut hormones such as cholecystokinin, peptide YY, and the incretins gastric inhibitory peptide and glucagon-like peptide 1 that potentiate insulin secretion from β-cells and are associated with regulation of food in-

take. The bioactive peptides also generated from whey protein may serve as endogenous inhibitors of DPP-4 in the proximal gut, preventing incretin degradation. Indeed, DPP-4 inhibitors were recently identified in whey protein hydrolysates. However, our study was not designed to test this. The lower glucagon levels found in all milk protein groups may be ascribed to increased GLP-1 levels which reduced glucagon and thereby lowered glucose production from the liver.

In conclusion, supplementing diet with milk protein fractions may improve glycemic indices in diabetic rats. The milk protein fractions had a differential impact on gene expression in normal and diabetic rats.

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■ Appendix

Table A1. Gene expression levels of selected genes in liver tissue of ZDF rats after 13 weeks of intervention with milk protein fractions

Gene	Experimental diet				
	Isolate	Hydrolysate	α-lactalbumin	Casein	Chow
Insulin sensitivity					
InsR	0.27 ± 0.09	0.25 ± 0.11	0.21 ± 0.07[c]	0.25 ± 0.06	0.33 ± 0.12
GLUT2	6.06 ± 1.30	6.46 ± 1.24	5.32 ± 1.00	5.10 ± 1.13[c]	6.65 ± 1.22
CPT-1	6.60 ± 2.12[f]	6.52 ± 2.11	4.10 ± 1.38	6.13 ± 1.12	5.40 ± 2.06
Irs1	0.16 ± 0.04	0.14 ± 0.05	0.14 ± 0.05	0.15 ± 0.04	0.16 ± 0.05
Irs2	0.24 ± 0.07	0.22 ± 0.08	0.20 ± 0.07	0.20 ± 0.06	0.25 ± 0.11
AKT	1.34 ± 0.34	1.18 ± 0.26	1.03 ± 0.40	1.07 ± 0.22	1.19 ± 0.32
AMPK	1.05 ± 0.25	0.93 ± 0.28	0.84 ± 0.20	1.03 ± 0.26	1.15 ± 0.30
AdipoR	0.42 ± 0.14	0.36 ± 0.13	0.32 ± 0.10	0.37 ± 0.10	0.46 ± 0.16
Inflammation					
CRP	26.35 ± 2.41[b]	22.69 ± 4.40[a]	22.50 ± 3.93	18.51 ± 2.13[e]	24.82 ± 3.04
Glucose					
GYS2	0.57 ± 0.16	0.58 ± 0.11	0.42 ± 0.11[g]	0.53 ± 0.12	0.83 ± 0.31
GADPH	15.89 ± 4.00[a]	13.65 ± 2.20	13.21 ± 3.06	11.49 ± 1.94	13.15 ± 4.10
G6Pase	6.65 ± 3.63	9.84 ± 5.54	7.95 ± 3.82	7.86 ± 2.90	11.04 ± 5.44
Transcription					
SREBP-1C	2.87 ± 1.32[a,c]	2.21 ± 0.80	2.16 ± 1.02	1.65 ± 0.65	1.51 ± 1.03
SREBP-2	0.31 ± 0.06[f]	0.28 ± 0.07[c]	0.21 ± 0.06[e]	0.26 ± 0.06[d]	0.38 ± 0.12
PPAR-α	2.00 ± 0.73	1.59 ± 0.52	1.28 ± 0.47	1.58 ± 0.55	1.87 ± 0.76
PPAR-γ	0.01 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.01
JNK	0.73 ± 0.16	0.70 ± 0.18	0.60 ± 0.15	0.64 ± 0.13	0.77 ± 0.21
Fat					
FAS	1.02 ± 0.39	0.85 ± 0.59	0.54 ± 0.16	0.60 ± 0.35	0.79 ± 0.59
LPL	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.03	0.02 ± 0.01	0.03 ± 0.01
LXR	2.24 ± 0.66	1.91 ± 0.70	1.64 ± 0.34	1.93 ± 0.40	2.17 ± 0.59

Legend: Data are means ± SD; n(ZDF) = 12/group, arbitrary units normalized to the geometric mean of 18S and Hprt1, and multiplied by 100. [a] $p < 0.05$, [b] $p < 0.0001$ vs. casein (one-way ANOVA). [c] $p < 0.05$, [d] $p < 0.01$, [e] $p < 0.0001$ vs. chow (one-way ANOVA). [f] $p < 0.05$ vs. α-lactalbumin (one-way ANOVA). [g] $p < 0.05$ vs. chow (Kruskal-Wallis nonparametric test).

Table A2. Gene expression level of selected genes in muscle tissue of ZDF rats after 13 weeks of intervention with milk protein fractions

Gene	Experimental diet				
	Isolate	Hydrolysate	α-lactalbumin	Casein	Chow
Insulin sensitivity					
AdipoqR	1.85 ± 0.53[d]	1.50 ± 0.41	1.30 ± 0.21	1.41 ± 0.39	1.57 ± 0.37
IR (InsR)	0.46 ± 0.14	0.51 ± 0.12	0.56 ± 0.11	0.55 ± 0.11	0.53 ± 0.15
Irs1	0.48 ± 0.21	0.49 ± 0.15	0.59 ± 0.17	0.55 ± 0.13	0.39 ± 0.10
Irs2	2.38 ± 1.88	2.26 ± 1.43	3.72 ± 2.41	2.51 ± 1.93	2.87 ± 2.50
AKT	5.41 ± 1.05	5.07 ± 0.68	4.86 ± 0.69	4.95 ± 0.54	5.68 ± 0.56
GLUT4	8.77 ± 2.39	8.39 ± 1.45	7.39 ± 1.57	8.77 ± 1.59	8.06 ± 1.10
AMPK	3.55 ± 1.61	2.88 ± 1.32	2.18 ± 0.35	2.60 ± 1.39	2.95 ± 13.30
CPT-1	0.13 ± 0.04	0.12 ± 0.03	0.12 ± 0.03	0.11 ± 0.02	0.12 ± 0.04
Glucose					
GADPH	132.90 ± 77.28	99.29 ± 69.24	61.76 ± 10.95	94.67 ± 63.61	81.83 ± 69.58
Transcription					
PGC-1α	0.41 ± 0.26	0.33 ± 0.13	0.65 ± 0.30	0.43 ± 0.18	0.65 ± 0.50
PPAR-γ	0.04 ± 0.01[b]	0.04 ± 0.01	0.04 ± 0.01	0.03 ± 0.01	0.03 ± 0.01
PPAR-α	0.54 ± 0.17	0.57 ± 0.12	0.61 ± 0.16	0.62 ± 0.14	0.63 ± 0.20
SREBP-1C	0.74 ± 0.19	0.75 ± 0.35	0.98 ± 0.18[c]	0.96 ± 0.25[c]	0.57 ± 0.17
JNK	0.98 ± 0.18	0.94 ± 0.14	0.89 ± 0.16	0.86 ± 0.10	1.02 ± 0.09
SREBP-2	0.41 ± 0.10	0.37 ± 0.05	0.34 ± 0.04	0.33 ± 0.09	0.36 ± 0.06
Fat					
LXR	0.21 ± 0.10[a,d]	0.15 ± 0.05	0.11 ± 0.02	0.13 ± 0.05	0.13 ± 0.05
FAS	0.04 ± 0.02	0.05 ± 0.04	0.04 ± 0.03	0.03 ± 0.01	0.03 ± 0.01
LPL	18.75 ± 8.27	24.28 ± 9.41	28.06 ± 3.14	25.91 ± 9.28	24.71 ± 10.21

Legend: Data are means ± SD; n(ZDF) = 12/group, arbitrary units normalized to the geometric mean of 18S and Hprt1, and multiplied by 100. [a] $p < 0.05$ vs. casein (one-way ANOVA). [b] $p < 0.05$, [c] $p < 0.01$ vs. chow (one-way ANOVA). [d] $p < 0.05$, [e] $p < 0.01$ vs. α-lactalbumin (one-way ANOVA).

Table A3. Gene expression level of selected genes in adipose tissue of ZDF rats after 13 weeks of intervention with milk protein fractions

Gene	Experimental diet				
	Isolate	Hydrolysate	α-lactalbumin	Casein	Chow
Insulin sensitivity					
IR (InsR)	0.41 ± 0.07	0.38 ± 0.09	0.42 ± 0.03	0.47 ± 0.10[g]	0.33 ± 0.08
GLUT4	1.32 ± 0.27[g]	1.03 ± 0.36[d]	1.17 ± 0.31[c,e]	1.62 ± 0.18[h]	0.77 ± 0.24
AMPK	0.26 ± 0.06[b]	0.21 ± 0.07[d]	0.26 ± 0.06[b]	0.36 ± 0.06[h]	0.22 ± 0.08
Irs1	0.46 ± 0.11	0.46 ± 0.11	0.50 ± 0.09	0.45 ± 0.07	0.40 ± 0.08
Irs2	1.35 ± 0.24	1.39 ± 0.38	1.36 ± 0.24	1.51 ± 0.26	1.19 ± 0.20
AKT	5.30 ± 1.17	5.00 ± 1.08	5.10 ± 0.56	5.37 ± 0.81	4.69 ± 1.02
Adipoq	41.59 ± 7.06	35.39 ± 12.53[c]	40.79 ± 7.02	51.89 ± 9.25[e]	39.75 ± 10.04
CPT-1	0.90 ± 0.19	0.91 ± 0.22	0.90 ± 0.23	0.77 ± 0.14	0.82 ± 0.19
Inflammation					
IL-6	0.02 ± 0.03	0.04 ± 0.07	0.03 ± 0.08	0.01 ± 0.01	0.01 ± 0.01
TNF-α	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01
Glucose					
GADPH	13.58 ± 1.93[e]	13.56 ± 2.50[e]	12.67 ± 1.37	12.60 ± 1.63	11.13 ± 1.93
Transcription					
PPAR-α	0.15 ± 0.03[a]	0.13 ± 0.05[b]	0.16 ± 0.06	0.20 ± 0.04[e]	0.14 ± 0.04
PPAR-γ	1.68 ± 0.28[f]	1.49 ± 0.42	1.48 ± 0.20	1.70 ± 0.35[f]	1.20 ± 0.24
JNK	2.17 ± 0.32	2.06 ± 0.43	2.18 ± 0.24	2.29 ± 0.34[e]	1.80 ± 0.38
SREBP-1C	1.91 ± 0.39	1.88 ± 0.64	1.85 ± 0.81	2.10 ± 0.47[e]	1.36 ± 0.46
SREBP-2	0.82 ± 0.19	0.77 ± 0.18	0.81 ± 0.16	0.70 ± 0.12	0.77 ± 0.21
Fat					
FAS	0.25 ± 0.88[d]	1.46 ± 0.49[d]	2.18 ± 0.87[d]	4.09 ± 1.11[h]	1.58 ± 0.79
LPL	124.90 ± 18.44	102.50 ± 31.43[b]	121.50 ± 16.34	140.20 ± 14.32	103.10 ± 24.50
LXR	3.28 ± 0.37[f]	3.11 ± 0.58	3.27 ± 0.34[e]	3.36 ± 0.41[f]	2.64 ± 0.51

Legend: Data are means ± SD; n(ZDF) = 12/group, arbitrary units normalized to the geometric mean of 18S and Hprt1, and multiplied by 100. [a] $p < 0.05$, [b] $p < 0.01$, [c] $p < 0.001$, [d] $p < 0.0001$ vs. casein (one-way ANOVA). [e] $p < 0.05$, [f] $p < 0.01$, [g] $p < 0.001$, [h] $p < 0.0001$ vs. chow (one-way ANOVA).

Table A4. Gene expression level of selected genes in liver tissue of Wistar rats after 13 weeks of intervention with milk protein fractions

Gene	Experimental diet				
	Isolate	Hydrolysate	α-lactalbumin	Casein	Chow
Insulin sensitivity					
InsR	0.30 ± 0.11	0.30 ± 0.19	0.34 ± 0.14	0.29 ± 0.010	0.26 ± 0.07
GLUT2	4.61 ± 1.48	4.99 ± 2.44	5.59 ± 1.34	4.27 ± 1.76	4.78 ± 1.05
CPT-1	4.31 ± 1.57	4.36 ± 1.94	4.51 ± 1.65	3.82 ± 1.23	4.27 ± 0.65
Irs1	0.20 ± 0.06	0.22 ± 0.13	0.22 ± 0.12	0.18 ± 0.06	0.16 ± 0.03
Irs2	0.42 ± 0.26	0.25 ± 0.06	0.29 ± 0.15	0.31 ± 0.17	0.44 ± 0.17
AKT	1.05 ± 0.31	4.57 ± 9.85	1.09 ± 0.35	0.92 ± 0.22	1.22 ± 0.13
AMPK	1.22 ± 0.41	1.15 ± 0.53	1.27 ± 0.38	1.15 ± 0.33	1.03 ± 0.25
AdipoqR	0.37 ± 0.12	0.45 ± 0.25	0.47 ± 0.20	0.39 ± 0.11	0.41 ± 0.07
Inflammation					
CRP	23.29 ± 4.49	28.62 ± 11.25	32.66 ± 7.17	23.34 ± 5.18	26.62 ± 6.12
Glucose					
GYS2	1.01 ± 0.42	1.04 ± 0.56	1.02 ± 0.32	0.90 ± 0.44	1.44 ± 0.70
GADPH	7.08 ± 1.34	6.88 ± 3.10	8.25 ± 3.16	6.33 ± 2.23	6.09 ± 1.56
G6Pase	7.88 ± 3.88	7.75 ± 4.06	8.91 ± 7.42	7.36 ± 1.49	7.52 ± 3.64
PDK4	0.40 ± 0.25	0.29 ± 0.14	0.36 ± 0.31	0.26 ± 0.20	0.48 ± 0.21
SDH	5.60 ± 1.22	5.10 ± 2.31	6.26 ± 1.80	5.67 ± 1.53	4.87 ± 0.44
Transcription					
PGC-1α	0.02 ± 0.01	0.01 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.01 ± 0.01
SREBP-1C	0.58 ± 0.20	0.76 ± 0.41	0.67 ± 0.32	0.66 ± 0.43	0.40 ± 0.18
SREBP-2	0.31 ± 0.05	0.44 ± 0.25	0.33 ± 0.13	0.23 ± 0.09[a]	0.40 ± 0.11
PPAR-α	1.34 ± 0.54	1.10 ± 0.71	1.26 ± 0.59	1.26 ± 0.51	1.11 ± 0.35
PPAR-γ	0.01 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.01 ± 0.01	0.01 ± 0.00
JNK	0.60 ± 0.17	0.65 ± 0.30	0.74 ± 0.26	0.59 ± 0.17	0.61 ± 0.10
Fat					
FAS	0.12 ± 0.08	0.16 ± 0.11	0.13 ± 0.09	0.07 ± 0.05	0.07 ± 0.04
LPL	0.04 ± 0.03	0.07 ± 0.04	0.06 ± 0.05	0.05 ± 0.02	0.08 ± 0.04
LXR	1.75 ± 0.44	2.30 ± 1.11	2.33 ± 0.62	1.98 ± 0.58	1.65 ± 0.41

Legend: Data are means ± SD; n(Wistar) = 8-9/group, arbitrary units normalized to the geometric mean of 18S and Hprt1, and multiplied by 100. [a] $p < 0.05$ vs. Chow (Kruskal-Wallis nonparametric test).

Table A5. Gene expression level of selected genes in muscle tissue of Wistar rats after 13 weeks of intervention with milk protein fractions

Gene	Experimental diet				
	Isolate	Hydrolysate	α-lactalbumin	Casein	Chow
Insulin sensitivity					
AdipoqR	1.48 ± 0.48	1.52 ± 0.48	1.43 ± 0.61	1.57 ± 0.92	1.56 ± 0.48
IR (InsR)	0.55 ± 0.18	0.54 ± 0.12	0.50 ± 0.15	0.54 ± 0.16	0.62 ± 0.15
Irs1	0.53 ± 0.29	0.73 ± 0.34	0.47 ± 0.14	0.52 ± 0.16	0.46 ± 0.04
Irs2	3.05 ± 2.22	2.00 ± 0.86	2.68 ± 1.48	3.60 ± 3.22	1.95 ± 0.84
AKT	5.47 ± 0.97	5.37 ± 0.87	5.28 ± 0.93	6.84 ± 5.26	6.23 ± 0.83
GLUT4	13.25 ± 3.08	11.48 ± 1.86	11.99 ± 2.16	18.37 ± 12.70	11.39 ± 2.15
AMPK	3.69 ± 1.58	3.71 ± 1.62	3.31 ± 1.51	3.66 ± 1.60	3.76 ± 1.49
CPT-1	0.14 ± 0.03	0.15 ± 0.05	0.13 ± 0.03	0.15 ± 0.07	0.17 ± 0.03
Glucose					
GADPH	123.30 ± 60.76	125.30 ± 69.11	113.50 ± 73.25	103.10 ± 63.77	108.50 ± 65.91
PDK4	15.02 ± 7.05	10.55 ± 4.24	9.20 ± 4.58	16.00 ± 10.90	12.23 ± 7.87
SDH	19.63 ± 3.06	18.51 ± 2.70	18.22 ± 2.90	26.41 ± 24.21	21.25 ± 4.03
Transcription					
PGC-1α	0.54 ± 0.17[a]	0.26 ± 0.09	0.55 ± 0.35	0.46 ± 0.28	0.48 ± 0.30
PPAR-γ	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.00
SREBP-1C	0.76 ± 0.41	0.83 ± 0.30	0.84 ± 0.37	1.24 ± 1.35	0.68 ± 0.20
PPAR-α	0.80 ± 0.19	0.59 ± 0.16	0.67 ± 0.14	0.82 ± 0.26	0.79 ± 0.14
JNK	1.09 ± 0.19	1.01 ± 0.19[b]	1.02 ± 0.18	1.13 ± 0.44	1.32 ± 0.15
SREBP-2	0.30 ± 0.05	0.29 ± 0.04	0.30 ± 0.04	0.38 ± 0.33	0.27 ± 0.07
Fat					
LXR	0.21 ± 0.09	0.21 ± 0.08	0.17 ± 0.07	0.20 ± 0.08	0.20 ± 0.16
FAS	0.03 ± 0.02	0.03 ± 0.01	0.03 ± 0.01	0.04 ± 0.02	0.03 ± 0.01
LPL	26.44 ± 15.32	26.14 ± 16.84	25.89 ± 12.48	39.52 ± 29.46	32.30 ± 13.52

Legend: Data are means ± SD; n(Wistar) = 8-9/group, arbitrary units normalized to the geometric mean of 18S and Hprt1, and multiplied by 100. [a] $p < 0.01$ vs. hydrolysate (Kruskal-Wallis nonparametric test). [b] $p < 0.05$ vs. chow (Kruskal-Wallis nonparametric test).

Table A6. Gene expression level of selected genes in adipose tissue of Wistar rats after 13 weeks of intervention with milk protein fractions

Gene	Experimental diet				
	Isolate	Hydrolysate	α-lactalbumin	Casein	Chow
Insulin sensitivity					
IR (InsR)	0.58 ± 0.15	0.51 ± 0.12	0.48 ± 0.09	0.58 ± 0.14	0.62 ± 0.11
GLUT4	2.29 ± 1.12	2.02 ± 0.84	2.89 ± 1.10[c]	3.77 ± 2.20[d]	1.34 ± 0.47
AMPK	0.31 ± 0.05	0.29 ± 0.07	0.32 ± 0.08	0.32 ± 0.09	0.23 ± 0.05
Irs1	0.84 ± 0.29	0.78 ± 0.23	0.76 ± 0.22	0.76 ± 0.22	1.03 ± 0.27
Irs2	2.72 ± 0.87	2.23 ± 0.58	2.15 ± 0.63	2.15 ± 0.77	2.47 ± 0.29
AKT	6.11 ± 0.91	6.47 ± 1.26	5.86 ± 1.04	5.70 ± 1.20	7.01 ± 1.10
Adipoq	82.97 ± 14.37	78.57 ± 8.98	84.44 ± 15.45	77.13 ± 11.02	83.47 ± 11.74
CPT-1	0.40 ± 0.08	0.38 ± 0.10	0.31 ± 0.08[b]	0.34 ± 0.09[a]	0.47 ± 0.06
Inflammation					
TNF-α	0.01 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
Glucose					
GADPH	10.19 ± 2.71	10.74 ± 2.51	11.43 ± 3.48	14.93 ± 8.53	7.87 ± 1.17
PDK4	1.62 ± 0.67	1.19 ± 0.38	1.37 ± 0.71	1.38 ± 0.73	1.47 ± 0.46
SDH	9.63 ± 1.56	9.50 ± 1.90	8.96 ± 0.70	10.10 ± 2.26	8.67 ± 1.35
Transcription					
PGC-1α	0.01 ± 0.00	0.01 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.01
PPAR-α	0.24 ± 0.08	0.21 ± 0.02	0.22 ± 0.04	0.24 ± 0.04	0.20 ± 0.04
PPAR-γ	2.10 ± 0.46	2.05 ± 0.39	1.86 ± 0.43	2.16 ± 0.23	2.28 ± 0.43
JNK	1.64 ± 0.26	1.62 ± 0.27	1.65 ± 0.26	1.65 ± 0.29	1.69 ± 0.22
SREBP-1C	4.06 ± 1.36	4.40 ± 0.82	4.49 ± 0.93	4.86 ± 1.03	4.22 ± 0.97
SREBP-2	0.44 ± 0.06	0.42 ± 0.07	0.44 ± 0.05	0.45 ± 0.07	0.45 ± 0.05
Fat					
FAS	6.47 ± 3.64	6.02 ± 3.83	12.85 ± 8.78	20.97 ± 18.25[c]	4.08 ± 2.60
LPL	160.10 ± 34.27	151.60 ± 31.41	160.80 ± 32.62	127.40 ± 26.68	151.20 ± 26.17
LXR	3.85 ± 0.73	3.72 ± 0.73	3.62 ± 0.58	3.78 ± 0.57	3.48 ± 0.61

Legend: Data are means ± SD; n(Wistar) = 8-9/group, arbitrary units normalized to the geometric mean of 18S and Hprt1, and multiplied by 100. [a] $p < 0.05$, [b] $p < 0.01$ vs. chow (one-way ANOVA). [c] $p < 0.05$, [d] $p < 0.01$ vs. chow (Kruskal-Wallis nonparametric test).

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