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Energy restriction prevents the development of type 2 diabetes in Zucker diabetic fatty rats: coordinated patterns of gene expression for energy metabolism in insulin-sensitive tissues and pancreatic islets determined by oligonucleotide microarray analysis

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Abstract

Energy restriction (ER) causes metabolic improvement in the prediabetic and diabetic state. Little information exists on the mechanism of action of ER, for example, on the changes at the transcriptional gene level in insulin-sensitive tissues. To gain further insight, we have investigated changes in gene expressions in skeletal muscle, liver, fat, and pancreatic islets after ER in male Zucker diabetic fatty rats. Eighteen Zucker diabetic fatty rats were divided at the age of 7 weeks into a control group (ad libitum diet) and an ER group (30% ER compared with the control group). Blood glucose, weight, and food intake were measured weekly. After 5 weeks, blood samples, and skeletal muscle, liver, visceral fat (epididymal fat pads), and islets tissue were collected. Gene expression was quantified with high-density oligonucleotide, microarray GeneChip technology. ER ameliorated the development of hyperglycemia, increased the levels of plasma insulin, and reduced plasma total cholesterol and the glucagon-insulin ratio ($P < .05$). In skeletal muscle, the expression of 55 genes increased and 245 decreased involving genes related to glucose metabolism (eg, phosphorylase kinase, pyruvate dehydrogenase kinase 4), lipid metabolism (eg, carnitine palmitoyltransferase 1, fatty acid transporter), and signaling pathways (eg, mitogen-activated protein kinases, protein kinase C). In the liver, the expression of 123 genes increased and 103 decreased involving genes related primarily to lipid metabolism. In pancreatic islets, the expression of 110 genes increased and that of 127 decreased, whereas in visceral fat, the expression of 279 genes increased and that of 528 decreased. ER counteracts the development of diabetes and causes changes in the expression of multiple genes involved in glucose and lipid metabolism in skeletal muscle, liver, and pancreatic islets, which may play an important role for the prevention of diabetes.

1. Introduction

In addition to a genetic component, type 2 diabetes is associated with environmental and lifestyle risk factors especially overweight and sedentary lifestyle. The development of type 2 diabetes can be reversed by lifestyle modifications such as dieting and exercise [1,2], and the symptoms decline or disappear in populations under starvation, for example, the food rationing imposed during the 1870 to 1871 siege of Paris [3,4].

Energy restriction (ER) is known to retard the aging process in rodents. Recently, it has been demonstrated that ER counteracts changes induced by aging at the level of gene expression in skeletal muscle of laboratory rodents [5,6]. However, no studies so far have elucidated the impact of prevention of type 2 diabetes by ER on gene expression.

The pathophysiology of type 2 diabetes is complex, and it is likely that a detailed analysis of gene expression patterns will reveal general as well as organ-specific alterations. Gluco- and lipotoxicity are considered to play a key role in the development of type 2 diabetes and its late

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complications [7-9]. Evidence indicates an increase in lipogenesis in extra-adipose tissues in obese subjects [10] and in animal models of type 2 diabetes [11-13]. When the maximal capacity to accumulate fat in the liver is reached, the diabetic phenotype occurs [11]. At least in part, increased intracellular reactive oxygen species (ROS) mediate glucotoxicity and possibly lipotoxicity in beta cells and other tissues [14,15]. ROS induce activation of stress-inducible genes, leading to organ failure and apoptosis [15].

Prospective studies assessing the physiological and biochemical effects of ER in humans can be difficult to carry out in a controlled setting. A suitable animal model may, however, provide a unique research tool for such studies. The Zucker diabetic fatty (ZDF) rats are leptin receptor–deficient, leading to the development of obesity, insulin resistance, and type 2 diabetes. The progression into overt diabetes is quite similar to human type 2 diabetes [16,17].

Our hypothesis was that prolonged ER causes alterations at the gene expression level in several metabolically important tissues that prevent the development of type 2 diabetes. The primary objective was to compare in male ZDF rats changes in the expression of genes involved in metabolic (glucose and lipid) and signaling pathways in skeletal muscles, liver, pancreatic islets, and visceral adipose tissue after 5 weeks of ER using the oligonucleotide microarray GeneChip technology.

The second objective was to evaluate possible differences in messenger RNA (mRNA) expression in various tissues of control and energy-restricted ZDF rats.

2. Research design and methods

2.1. Animals

Male ZDF (Gmi-*fa/fa*) rats were purchased from Genetic Models (Indianapolis, IN). Animals were housed in individual cages in a vivarium, which maintains a constant temperature and a 12-hour light-dark cycle. Animals were fed with commercial chow (Purina Formulab 5008, Genetic Models). Tap water was given ad libitum.

2.2. Experimental protocol

After acclimatization, 18 animals at the age of 7 weeks were randomly divided into 2 groups, a control group (n = 9) and an ER group (n = 9). The control animals were maintained on ad libitum diet. The animals in the ER group were fed a 30% energy-restricted diet compared with the ad libitum fed group. A 30% ER was chosen because lean Zucker rats (non–leptin receptor–deficient) fed ad libitum eat about 30% less than ZDF rats. The dietary regimen supplied an adequate daily intake in minerals, vitamins, and energy for the animals in the ER group. The 2 groups were studied in parallel for a period of 5 weeks (from 7 to 12 weeks of age). All institutional guidelines for care and use of animals were followed. Once per week, animals were weighed in the morning, and fasting blood glucose was measured in blood from the tail vein (see below). Food consumption by ad libitum–fed animals was determined weekly by weighing out 50 g of food and 24 hours later weighing the remainder in the tray.

At the end of the fifth week, after 4 hours without access to food, the animals were anesthetized with pentobarbital (50 mg/kg IP), and capillary blood samples were obtained from the retro-ocular plexus using a 75-μL heparinized capillary tube. Successively, pancreas, liver, skeletal muscle (soleus), and visceral fat tissues (epididymal fat pads) were isolated immediately. Liver, skeletal muscle, and fat tissues were rapidly frozen and stored at -80°C.

2.3. Biochemical measurements

The weekly control of blood glucose concentration was carried out using One Touch Instrument (Lifescan, Milpitas, CA). Other blood samples were placed on ice and centrifuged (10 minutes, 4°C, 4000 rpm). Total cholesterol was analyzed with the cholesterol CHOD-PAP method (Roche, Mannheim, Germany). Triglyceride was analyzed with the triglyceride GPO-PAP method (Roche). Free fatty acid was analyzed with NEFA C kit, ACS ACOD method (Wako, Neuss, Germany). Plasma insulin and glucagon were analyzed by RIA kits (Linco Research, St. Charles, MO).

2.4. Isolation of pancreatic islets

Islets were isolated by the collagenase digestion technique [18] with minor modifications. In brief, when animals were anesthetized, a midline laparotomy was performed. The pancreas was retrogradely filled through the pancreatic duct with 9 mL ice-cold Hanks balanced salt solution (Sigma, St. Louis, MO) supplemented with 1 mL of 0.9 mg/mL collagenase P (Boehringer Mannheim, Mannheim, Germany). The pancreas was subsequently removed and incubated for 17 minutes at 37°C in a shaking water bath. After rinsing in ice-cold Hanks balanced salt solution, the islets were handpicked under a stereomicroscope and stored in 1.5 mL Trizol (Gibco, Roskilde, Denmark).

2.5. mRNA preparation and analysis

2.5.1. RNA extraction and sample pools

Total RNA was isolated from the different tissues using Trizol. For GeneChip analysis, an equal amount (10 μg) of total RNA from a given tissue was pooled in 2 subgroups (RNA from 4 to 5 animals in each subgroup).

2.5.2. Gene expression chip analysis

RNA labeling, array hybridization, and scanning were performed as previously described 19 according to the Affymetrix (Santa Clara, Calif) technical manual. All arrays were visualized using Affymetrix Genechip 5.0 software. The Rat Genome U34 A (Santa Clara, CA) monitors

the expression of more than 8000 genes and expressed sequence tag EST), clusters.

2.5.3. Data analysis

In total, 16 gene chips were used, 2 biological replicas for each tissue and condition. After global scaling, the signal, the detection (present, P; absent, A), the signal log ratio (SLR; ie, the logarithm in base 2 of the fold change), and the different call change (I, increase; D, decrease; M, moderate I or D; NC, no change) were calculated. For every tissue, we compared the gene expression of the intervention groups vs the control group in a double-cross analysis (concordance analysis), for a total of 4 comparisons. Moreover, for every gene or EST, we calculated the concordance on the different call change (I + MI, increase and moderate increase; D + MD, decrease and moderate decrease; or NC, no change), the average fold change, and the average SLR. As cutoff value, concordance in the different call change of 75% or more was chosen. Finally, genes with a mean SLR of 0.8 or more or of −0.8 or less (arbitrarily chosen) were considered up- or down-regulated, respectively, and were tentatively grouped with respect to the putative functions.

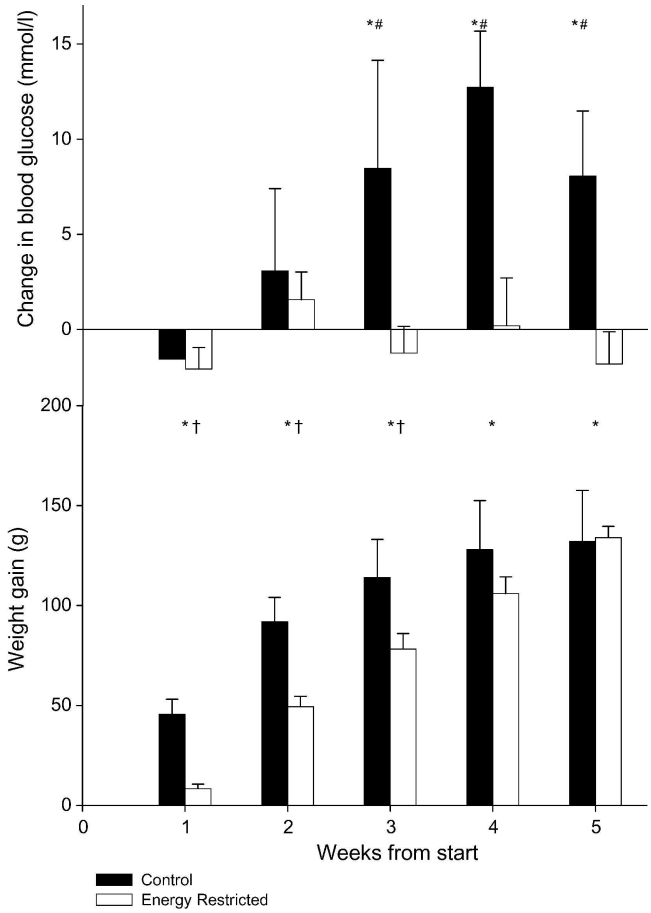

Fig. 1. Dynamic change in blood glucose and weight gain. Bars represent the difference from baseline and are expressed as means ± SD. *$P < .001$, significant difference from base line; #$P < .001$, significant difference from corresponding value in energy-restricted group.

Table 1
Effects of 5 weeks' ER (30%) on metabolic parameters in male ZDF rats (mean ± SE)

	Control (n = 9)	ER (n = 9)
Fasting glucose (mmol/L)	12.6 ± 0.9	2.9 ± 1.1**
Weight gain (g)	132 ± 4	134 ± 0.9
Insulin (ng/mL)	9.6 ± 0.4	12.8 ± 0.2*
Glucagon (pg/mL)	63 ± 6	57 ± 4
Glucagon-insulin ratio	6.9 ± 0.6	4.5 ± 0.3*
Triglycerides (mmol/L)	6.8 ± 0.3	6.6 ± 0.3
Free fatty acids (mmol/L)	2.3 ± 0.2	2.4 ± 0.1
Cholesterol (mmol/L)	3.9 ± 0.2	3.0 ± 0.0*
Urine glucose (mmol/L)	106.7 ± 20.4	0.9 ± 0.0*

* $P \leq .05$, significantly different from control after 5 weeks.
** $P \leq .01$, significantly different from control after 5 weeks.

2.6. Statistical analysis

For metabolic measurements, statistical analysis was performed using 2-way analysis of variance or unpaired or paired Student *t* test, as appropriate. Data are expressed as the mean ± SEM or otherwise specified. Differences were considered significant at *P* values less than .05. GeneChip analyses were presented as specified above. Detection signal was only specified in the text if appropriate.

3. Results

3.1. Metabolic profiles

At the start of the intervention study, no significant difference was measured in body weight and blood glucose between the groups. Blood glucose was 4.6 ± 0.5 mmol/L in the control group and 4.7 ± 0.6 mmol/L in the energy-restricted group, whereas the weight was 221.8 ± 4.1 g in the control group and 227 ± 7.7 g in the energy-restricted group. The time course of changes in the glucose level and the weight gain during the 5 weeks are presented in Fig. 1. After 5 weeks, the animals in the control group (n = 9) exhibited hyperglycemia (average blood glucose level, 12.6 ± 0.9 mmol/L) compared with the animals (n = 9) in the ER group (average blood glucose level, 2.9 ± 1.1 mmol/L; $P < .0001$). As seen in Table 1, total cholesterol concentrations and the glucagon-insulin ratio were higher in control than in energy-restricted rats, whereas insulin concentration was lower in control compared with energy-restricted rats. The weight gain was identical in the 2 groups at the end of the 5 weeks; however, the dynamic changes in weight gain were different (Fig. 1).

3.2. GeneChip analysis

We examined the possibility that changes in mRNA expression of genes and ESTs present in the Affymetrix Genechip RG-U34A might account for the beneficial effects of ER. In Tables 2-7, the mRNA expression changes with a concordance in different call of 75% or more and an SLR of 0.8 or more or −0.8 or less and a putative function considered

to be associated either to metabolism (glucose and lipid), signaling, and transcription are shown. Information on differentially expressed genes belonging to other functional groups is available at the web site: http://www.mdl.dk/SupplDataPub.htm.

3.2.1. Skeletal muscle mRNA expression

Of all the genes or ESTs surveyed in the oligonucleotide microarray, 7566 (86%) displayed a concordance of 75% or more. Of those, 55 (0.7%) increased expression, 245 (3.3%) decreased expression, and 7266 did not change expression among the ER group vs the control group. As described in Research Design and Methods, we grouped the genes that were up-regulated (SLR $\geq$ 0.8) or down-regulated (SLR ≤ -0.8) according to their putative function (Tables 2 and 3, Supplementary Tables 1 and 2).

In the ER group, we found that the expression of a number of genes involved in formation of proteins, lipid transport (eg, fatty acid transporter, acyl-coenzyme A [CoA] synthetase, carnitine palmitoyltransferase 1), β-oxidation (eg, acyl-CoA dehydrogenase, very long-chain, dodecenoyl-CoA δ isomerase, mitochondrial 2,4-dienoyl CoA reductase 1), and in fatty acid and triglyceride synthesis (eg, acetyl-CoA carboxylase β and diacylglycerol acyltransferase) was down-regulated in skeletal muscle tissue. However, not all findings were ambiguous, for example, the expression of genes considered involved in lipid metabolism (eg, Spot 14 and stearyl-CoA desaturase mRNA) was up-regulated. Moreover, the gene expression of 4 enzymes involved in glucose metabolism was down-regulated (Table 2). Genes involved in formation of protein kinases (eg, p38 mitogen-activated protein kinase [MAPK], stress-activated protein kinase α2) were down-regulated. The expression of 9 genes coding for transcription factors was also changed (Table 5).

Table 2
Genes influencing the metabolism of glucose and lipids in skeletal muscle

GenBank	Title	SLR
AA893242	Acyl-CoA synthetase long chain	−2.74
AB004329	Acetyl-coenzyme A carboxylase β	−2.46
X98225	α Subunit of mitochondrial trifunctional protein	−2.41
M33648	Mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase	−2.35
D21869	Phosphofructokinase-M	−2.26
AF030163	Uncoupling protein 3 mitochondrial	−2.16
X51415	Hormone-sensitive lipase	−1.95
U50842	Ubiquitin ligase (Nedd4) protein	−1.94
AF063302	Carnitine palmitoyltransferase	−1.83
X13722	LDL receptor	−1.82
AF034577	Pyruvate dehydrogenase kinase isoenzyme 4	−1.68
X62841	Potassium channel protein	−1.57
M92919	Phosphorylase kinase α-subunit	−1.51
X76988	Sialyltransferase 5	−1.47
U20195	Phosphoglucomutase	−1.46
D30647	Acyl-CoA dehydrogenase very long chain	−1.33
AF080568	Phosphate cytidylyltransferase 2 ethanolamine	−1.29
D00569	2,4-Dienoyl CoA reductase 1 mitochondrial	−1.12
AA859529	Diacylglycerol acyltransferase	−0.96
AI172017	Aldehyde dehydrogenase 2 mitochondrial	−0.85
U78977	ATPase class II type 9A	−0.85
AB005743	Fatty acid transporter	−0.85
AA998446	Phosphotidylinositol transfer protein β	−0.83
AI170568	Dodecenoyl-CoA δ isomerase (3,2-*trans*-enoyl-CoA isomerase)	−0.82
U83880	Glycerol-3-phosphate dehydrate dehydrogenase	0.86
M10934	Retinol-binding protein	1.14
K01934	Spot 14	3.21
J02585	Stearyl-CoA desaturase	4.56

This table as well as Tables 3-7 lists the up- and down-regulated genes in the energy-restricted ZDF rats as compared with controls. The first column lists the GenBank accession number for the respective genes. The second column lists the name of either the gene or the gene product. The third column lists the mean of the relative expression intensity values, expressed as SLR (see Research Design and Methods), of the group on ER compared with control. The present table shows genes with the putative function considered to be related with metabolism, signaling, or transcription/nuclear factor. A complete list is available on the web site: http://www.mdl.dk/publications_sup.htm.

Table 3
Genes influencing signaling in skeletal muscle

GenBank	Title	SLR
AF068202	A kinase (PRKA) anchor protein 1	−1.52
X74293	α 7A integrin	−0.91
U31816	Calcium channel α-1S subunit (ROB1)	−4.47
X53363	Calreticulin	−1.42
AJ005984	Endosulfine α	−0.87
AI011376	S78100 mitogen-activated protein kinase–activated protein kinase	−1.02
X74402	GDP-dissociation inhibitor 1	−0.84
AA817892	Guanine nucleotide binding protein β2 subunit	−0.84
M15481	Insulin-like growth factor 1	−1.82
AA963674	Mitogen-activated protein kinase kinase 2	−1.06
S61973	NMDA receptor glutamate-binding subunit	−1.43
AI137862	p38 Mitogen-activated protein kinase	−0.92
Y08355	PKC-ζ–interacting protein	−1
D17521	Protein kinase C–regulated chloride channel	−1.08
S74351	Protein tyrosine phosphatase	−1.49
U57500	Protein tyrosine phosphatase α	−1.05
U57501	Protein tyrosine phosphatase γ	−2.43
U02553	Protein tyrosine phosphatase nonreceptor type 16	−1.08
L19933	Protein tyrosine phosphatase receptor type D	−1.53
X12535	ras-related protein p23	−1.12
D14418	Regulatory subunit of protein phosphatase 2A	−0.98
X96488	SAP kinase-3	−1.53
L27112	Stress-activated protein kinase α2	−1.1
AF039085	Synaptogyrin 2	−1.42
U63923	Thioredoxin reductase 1	−0.83
AJ012603	Tumor necrosis factor α–converting enzyme	−1.53
AI237654	Up-regulated by 1,25-dihydroxyvitamin D-3	−1.16
AA799729	Phosphodiesterase 4B cAMP-specific	0.92

3.2.2. Liver mRNA expression

Of all the genes or ESTs surveyed in the oligonucleotide microarray, 8085 (89%) displayed a concordance of 75% or more. Of those, 123 (1.5%) had increased expression, 103 (1.3%) decreased expression, and 7859 did not change expression (Supplementary Tables 3 and 4). We found that most of the changes in mRNA expression were related to metabolic pathways (Table 4). Especially genes involved in lipid metabolism were differentially changed. In particular,

Table 4
Genes influencing the metabolism in the liver

GenBank	Title	SLR
Y09333	Mitochondrial very long-chain acyl-CoA thioesterase	−1.58
AB017260	Carnitine transporter solute carrier family 22 (organic cation transporter) member 5	−1.33
M96601	Taurine/β-alanine transporter	−1.27
D10262	Choline kinase	−1.1
U08976	Enoyl hydratase	−1.02
D00569	2,4-Dienoyl CoA reductase 1, mitochondrial	−1.01
AI178971	Hemoglobin α1	−0.99
X07259	Cytochrome *P*-452	−0.97
X60328	Cytosolic epoxide hydrolase	−0.93
AI170568	Dodecenoyl-CoA δ isomerase	−0.86
U07971	L-Arginine glycine amidinotransferase	−0.82
J05210	ATP citrate lyase	0.82
M64755	Cysteine-sulfinate decarboxylase	0.93
M76767	Fatty acid synthase	0.93
E01524	Soluble NADPH-cytochrome *P*450 reductase	0.96
AI008020	Cytosolic malic enzyme	1.02
X05684	Pyruvate kinase liver	1.17
AI105137	Glutathione *S*-transferase mitochondrial	1.32
M10068	*P*450 (cytochrome) oxidoreductase	1.32
X91234	17-β Hydroxysteroid dehydrogenase type 2	1.39
S69874	Cutaneous fatty acid–binding protein	1.42
J05035	Steroid-5-α-reductase α polypeptide 1 (3-oxo-5 α-steroid δ 4-dehydrogenase α1)	1.44
AA945573	Cytochrome *P*450 2c39	1.47
J03863	Serine dehydratase	1.59
AI175764	Liver stearyl-CoA desaturase	1.83
X13119	Serine dehydratase	2.09
M26594	Malic enzyme	2.12
AF036761	Stearyl-CoA desaturase 2	2.47
AA926149	Catalase	2.49

the mRNA expression of key enzymes involved in lipid synthesis was up-regulated by ER (eg, malic enzyme, stearyl-CoA desaturase, and fatty acid synthase), whereas the gene expression of a number of enzymes involved in lipid oxidation was down-regulated (mitochondrial very long-chain acyl-CoA thioesterase, 3,2-*trans*-enoyl-CoA isomerase). Moreover, some genes coding for transcription factors were regulated in parallel in liver and skeletal muscle tissue (eg, Egr1, Arntl) (Table 5).

3.2.3. Visceral adipose tissue mRNA expression

Of all the genes or ESTs surveyed in the oligonucleotide microarray, 8096 (92%) displayed a concordance of 75% or more, and of those, 279 (3.4%) increased expression, 528 (6.5%) decreased expression, and 6349 did not change (Table 6, Supplementary Tables 5 and 6).

3.2.4. Islet mRNA expression

Of the genes or ESTs surveyed in the oligonucleotide microarray, 110 (1.3%) increased expression, 127 (1.6%) decreased expression, and 7688 did not change expression in the energy-restricted group compared with the control group. Seventy-nine pairs satisfied our criteria (Table 7 and Supplementary Table 7). Genes coding molecules involved

Table 5
Genes influencing nuclear factors in the liver, skeletal muscle, and pancreatic islets

GenBank	Title	SLR		
		Liver	Skeletal muscle	Pancreatic islets
AF015953/ AB012600	Aryl hydrocarbon receptor nuclear translocator–like (BMAL1b)	−3.43	−1.8	
M18416	Early growth response 1	−1.28	−0.94	
U75397	Krox-24	−1.2		
AF023087	Nerve growth factor–induced factor A	−1.11		
S77528	C/EBP-related transcription factor	0.8		
AB016532	Period homolog 2	0.95	1.29	
AF072439	Zinc finger protein 37	1.05		
AA900476	Cbp/p300-interacting transactivator	1.15		
J03179	D site albumin promoter–binding protein	1.52	1.68	2.41
AA892801	Eukaryotic translation elongation factor 2		−2.9	
M61725	Transcription factor UBF2		−2.27	
X06769	c-*fos*		−1.97	
AF003008	Max interacting protein 1		−1.38	
U17254	Immediate early gene transcription factor NGFI-B		−1.13	
AF020618	Progression elevated gene 3		−1.11	
U05014	Eukaryotic translation initiation factor 4E binding protein 1		−1.02	
AF087437	PEBP2 β		0.8	
U20796	Nuclear receptor Rev-ErbA-β		0.86	
U64705	Protein synthesis initiation factor 4AII gene			−1.17
AF096835	Eukaryotic translation initiation factor 2 α kinase 3			−1.02
AA900476	Cbp/p300-interacting ransactivator, with Glu/Asp-rich carboxy-terminal domain 2			−0.87
AI177986	Eukaryotic initiation factor 5 (eIF-5)			−0.83
AF009329	*Rattus norvegicus* enhancer-of-split and hairy-related protein 1 (SHARP-1) mRNA, complete cds			0.93
AF004431	NK6 transcription factor related, locus 1 (*Drosophila*)			1.34

Table 6
Genes influencing metabolism and signaling in pancreatic islets

	GenBank	Title	SLR
Metabolism	M33648	Rat mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase	−2.73
	X02291	Aldolase B	−2.28
	AA925752	CD36 antigen (collagen type I receptor, thrombospondin receptor)	−2.15
	AI232096	Solute carrier family 15 (H+/peptide transporter), member 2	−1.35
	D90404	Cathepsin C (dipeptidyl peptidase I)	−1.28
	U07971	L-Arginine glycine amidinotransferase	−1.2
	U90829	APP-binding protein 1	−1.12
	J02791	Acyl-coenzyme A dehydrogenase, C-4 to C-12 straight-chain	−1.07
	D89069	Carbonyl reductase	−0.97
	M15185	*S*-adenosyl-L-homocysteine hydrolase	−0.96
	U38379	γ-Glutamyl hydrolase	−0.89
	AI230614	ATPase Na^+/K^+ transporting β1 polypeptide	−0.8
	M83746	Proprotein convertase subtilisin/kexin type 2	0.94
	S40669	Type 2 proinsulin processing endopeptidase = subtilisin homolog	1.12
	AA893325	Ornithine aminotransferase	2.12
Signaling	M35299	Serine protease inhibitor, kanzal type 1/trypsin inhibitor–like protein, pancreatic	−1.84
	M25890	Somatostatin	−0.93
	S78218	Protein phosphatase 1β	−0.9
	K02248	Somatostatin-14	−0.81
	X80477	Purinergic receptor P2X, ligand-gated ion channel 1	−0.81
	M36317	Thyrotropin-releasing hormone (TRH) precursor	0.93
	J05592	Protein phosphatase 1, regulatory (inhibitor) subunit 1A	1.13
	M98049	Pancreatitis-associated protein 1	2.27

in protein synthesis were up-regulated, for example, endopeptidase. The mRNA expression of amyloidogenic glycoprotein, immune response molecules, was down-regulated. mRNA-coding somatostatin was also down-regulated.

4. Discussion

We demonstrated that 5 weeks of ER counteracts the development of diabetes in ZDF rats and is concomitantly associated with changes in the expression of genes involved in metabolic pathways in skeletal muscles (eg, down-regulation of phosphorylase kinase, pyruvate dehydrogenase kinase isoenzyme 4, and enzymes involved in the β-oxidation), liver (eg, up-regulation of enzymes involved in fatty acid synthesis), visceral adipose tissue (eg, up-regulation of carnitine palmitoyltransferase 1), and pancreatic islets (eg, gene expression of molecules involved in the synthesis of protein). Furthermore, changes in the expression of new genes that may influence the control of diabetes have been pointed out (eg, MAPK and 3-hydroxy-3-methylglutaryl CoA [HMG-CoA] synthase). The limitations of microarray technology and the methodologies to analyze the results have previously been discussed [20-24].

The GeneChip data demonstrated that ER differentially modified the transcription of several genes related to various functions in tissues examined. Our data indicate that regulation on the gene expression level causes tissue adaptation to ER that is likely to be involved in the prevention of type 2 diabetes.

High-density oligonucleotide microarray provides a powerful tool for studying parallel changes in expression of a large number of genes, and Affymetrix Genechip RG-U34A microarray has been used extensively [19,25-30]. Thus, we have considered high-density oligonucleotide microarray analysis to be a suitable method to compare changes in metabolic pathways at the mRNA level and to identify candidate genes in different tissues related to the

Table 7
Selected genes influencing metabolism and signaling in visceral fat

	GenBank	Title	SLR
Metabolism	U02096	Fatty acid–binding protein 7, brain	1.18
	AB009463	Low-density lipoprotein receptor–related protein 3	1.24
	V01235	Fatty acid–binding protein 1 (liver)	1.64
	AA925752	CD36 antigen (collagen type I receptor, thrombospondin receptor)	2.32
	S52878	Fatty acid–binding protein	4.58
	L07114	Apolipoprotein B editing protein	6.61
Signaling	J04636	Nicotinic acetylcholine receptor β-3 subunit precursor	−2.2
	U04319	Interleukin 1 receptor–like 1 (Fos-responsive gene 1)	−2.04
	AA945907	Atrial natriuretic peptide clearance receptor 3	−2.02
	U04317	Interleukin 1 receptor–like 1 (Fos-responsive gene 1)	−1.98
	X96488	SAP kinase-3	−1.91
	S46785	Insulin-like growth factor–binding protein	−1.8
	S79676	Interleukin 1β–converting enzyme	−1.77
	AF013144	*R norvegicus* mitogen-activated protein kinase phosphatase (cpg21) mRNA, complete cds	−1.72
	AJ005424	Mitogen-activated protein kinase 7	−1.66
	L04485	Mitogen-activated protein kinase kinase 2	−1.64
	AA850734	Vascular endothelial growth factor	−1.61
	AJ011115	Endothelial nitric oxide synthase	−1.56

prevention of type 2 diabetes after ER. We did not study the changes in the metabolic pathways at the protein levels or the allosteric control of the proteins in the present study.

4.1. Metabolic changes

The 30% ER potently counteracted the development of diabetes in ZDF rats. It improved blood glucose, plasma cholesterol, and the glucagon/insulin ratio, as previously shown [31-33]. It is noteworthy that the 2 groups had the same body weight but different temporal weight gains after 5 weeks (Fig. 1). At the end of the experiment, the control animals had glucosuria (Table 1). The concomitant energy loss in the control group may well explain that the nondiabetic ER group attained a weight not significantly different from that of the other groups. We cannot exclude a different fat distribution in control animals and ER animals because we did not measure total fat and skeletal muscle mass.

An extramuscular defect in fatty acid metabolism could contribute to the triglyceride accumulation in skeletal muscle and the lipotoxic effects in skeletal muscles seen in obesity and type 2 diabetes [34,35]. In addition, the liver plays an important role in the development of type 2 diabetes. However, a diabetic phenotype occurs only when the hepatic capacity to accumulate fatty acids is reached [11]. In accordance with this, ER down-regulated mRNA-coding enzymes and molecules involved in lipid metabolism (lipid transport, β-oxidation, and triglyceride accumulation) in skeletal muscle (Table 3). However, in the liver, ER up-regulated genes involved in fatty acid synthesis (malic enzyme, stearyl-CoA desaturase, and fatty acid synthase) (Table 4), but down-regulated genes involved in β-oxidation (Table 5). All together, this indicates that the capacity to accumulate triglycerides is maintained, whereas the formation of ROS is probably decreased in the liver of energy-restricted ZDF rats (Supplementary Fig. 2B). Skeletal muscle also experienced an up-regulation of Spot 14, which has been associated with lipid metabolism but with unknown function, and of the mRNA expression of stearyl-CoA desaturase, which catalyzes the introduction of a double bond in fatty acids [36]. Phospholipid and cholesterol ester compositions are important to preserve cell membrane fluidity and function, and the up-regulation of stearyl-CoA desaturase mRNA may reflect a beneficial adaptation (Supplementary Table 2). Uncoupling protein 3 was also down-regulated in skeletal muscle. Uncoupling protein 3 could play a role in the regulation of fatty acid ion in the mitochondria [37,38], and it is therefore tempting to speculate that uncoupling protein 3 may link fatty acid metabolism and the activation of stress-activated pathways (Supplementary Fig. 2). Skeletal muscle of energy-restricted ZDF rats experienced a down-regulation of the expression of 2 genes coding for enzymes directly involved in glucose metabolism (Table 2) and 2 genes coding for enzymes that indirectly regulate glucose metabolism (Table 3). In liver, only PK-L was regulated (up) in energy-restricted animals. Similar differences in gene expression were found between nondiabetic and diabetic rodents [39,40], which supports these results. These changes point to an increase in hepatic glycolysis and an increase in skeletal muscle glycogen synthesis in nondiabetic energy-restricted ZDF rats (Supplementary Fig. 2).

In adipose tissue, mRNA for fatty acid–binding protein and low-density lipoprotein receptor–related protein 3, all involved in the kinetics of lipids, was unregulated in energy-restricted ZDF rats. However, despite the high number of genes changed, any changes in gene expression of specific metabolic pathways were modified in a complementary manner in visceral adipose tissue.

4.2. Signaling

Increased ROS formation is associated with increased activity of protein kinase C (PKC), MAPK, and NH_2-terminal Jun kinases/stress-activated protein kinases during the development of type 2 diabetes and diabetic complications [9,15]. In accordance with this, ER down-regulated the gene expression of stress-activated kinases, for example, p38 MAPK, SAP kinase-3 (Table 3), and PKC-regulated chloride channel (Table 3) in skeletal muscle tissue. Moreover, we showed that skeletal muscle of energy-restricted ZDF rats also saw a down-regulation of protein tyrosine phosphatases, which directly deactivate the insulin receptor [41] and are involved in a variety of signaling pathways including the stress-activated pathways [42] (Table 3). PKC-interactive protein, which inhibits glucose transporter (GLUT)-4 mobilization to the plasma membrane through the inhibition of PKC ζ/λ [43,44], was also down-regulated at the gene expression level. Another mechanism known to induce the activation of stress-activated pathways is the formation of several oxygenated 12-lipooxygenase products [15]. mRNA of 12-lipooxygenase was down-regulated in the liver of energy-restricted ZDF rats, as was also the tumor necrosis factor (TNF) I receptor, which is one of the factors linked to obesity-induced insulin resistance [45]. In skeletal muscles, TNF-converting enzyme [46], which generates the soluble TNF forms, was concomitantly down-regulated.

mRNA of molecules involved in signaling was considerably changed in adipose tissue of energy-restricted ZDF rats. In accordance with the hypothesis that diabetes prevention, by means of ER, influences the gene expression of molecules involved in the MAPK pathways, mRNA of MAPK 7, MAPK kinase 2, and SAP kinase-3 was down-regulated in adipose tissue of energy-restricted ZDF rats. We observed down-regulation of genes involved in development of cardiovascular complications, indicating that adipose tissue is not just involved in accumulation of triglycerides [47]. Because we applied a whole fat pad to isolate RNA, it is not known whether the elevated expression of genes associated with inflammation in control

rats was caused by an enrichment of proinflammatory cells or the result of elevated gene expression in adipocytes.

4.3. Nuclear factors

The nuclear factors, transcription factors, and other intranuclear proteins coordinate the transcription and translation of mRNA. We found that ER was able to coordinate the transcription of several nuclear factors in different tissues, for example, mRNA of early growth response 1 (erg-1) was down-regulated in skeletal muscle (Table 3) and liver (Table 5). An explanation could be that erg-1, c-*fos*, and progression elevate gene 3 (all down-regulated at the mRNA level in energy-restricted ZDF rats) may mediate the effects of stress-induced pathways at the transcriptional levels.

Alterations in mRNA of circadian clock transcription factors and molecules, such as D site albumin promoter–binding protein (DBP) and period homolog 2 (PER-2) in the heart, have been found to be different between control and streptozocin diabetic rats [40]. Corroborating this, we found that mRNA coding for DBP, PER-2, or aryl hydrocarbon receptor nuclear translocator–like (Arnt-1/Bmal-1) was differentially regulated in energy-restricted ZDF rats compared with hyperglycemic ZDF rats. Moreover, the mRNA of DBP and PER-2 was up-regulated in parallel in liver skeletal muscle and islets (Tables 2-6 and Supplementary Table 2).

4.4. Pancreatic islets

We found increased plasma insulin levels in energy-restricted ZDF rats compared with the hyperglycemic ZDF rats (controls) in accordance with previous reports [48]. In a previous report [17], no change in islet insulin mRNA levels was found in ZDF rats compared with control. In the present study, gene expression of molecules involved in protein synthesis, for example, proprotein convertase subtilisin/kexin type 2, was not regulated in energy-restricted animals. A decrease in the phosphorylation of specific protein seems necessary for glucose-mediated insulin secretion to take place [49]. We found that protein phosphatase β was down-regulated in energy-restricted ZDF rats, whereas protein phosphatase 1 regulatory (inhibitor) subunit 1A was up-regulated, which suggests a conservation of glucose-induced insulin secretion in islets of energy-restricted rats.

Islet amyloid correlates with the duration and the severity of type 2 diabetes [50]. In accordance with this, the mRNA of amyloidogenic glycoprotein was down-regulated in energy-restricted ZDF rats (Supplementary Table 7).

ER influences the immune system activation, for example, interleukin 15, which we found down-regulated in islets (Table 7). Beta cells have been considered to be very sensitive to oxidative stress [50]. At the mRNA level, however, we found that ER did not cause changes in the oxidative stress-related pathways or in enzymes known to prevent the damage induced by ROS. An increase in inflammatory and immune responses with degeneration of beta cell and abnormal glucose homeostasis may cause apoptosis and beta cells loss. Recently, however, it was reported that interleukin 15 fails to counteract functional suppression of beta cells by cytokines [51].

Mitochondrial HMG-CoA synthase is the first and rate-limiting enzyme for the production of ketone bodies. Interestingly, we found that in pancreatic islets after ER, mRNA for HMG-CoA synthase was down-regulated. In rats, HMG-CoA synthase has been found in different organs, such as liver, testis, and colon, but only in low amounts in pancreas. The presence of HMG-CoA synthase mRNA and the possible activation of ketone body formation in pancreatic islets and skeletal muscle are puzzling. Ketones can directly influence the release of insulin and somatostatin from the endocrine pancreas [52]. Thus, islet ketones may have a direct action on islet hormone secretion. Evidence has been put forward that increased HMG-CoA synthase could be associated with nonfunctioning islets [53]. Caution should, however, be exercised in relation to isolated islets because the isolation procedure may influence gene expressions.

4.5. Conclusion

The present study has demonstrated that ER prevents the development of hyperglycemia in ZDF rats and is associated with changes in expression of mRNA levels of genes involved in signaling pathways (eg, MAPKs) and energy metabolism in muscles. In the liver, an up-regulation of mRNA of genes involved in fatty acid synthesis was detected. In islets, a major finding was that HMG-CoA synthase was down-regulated in energy-restricted animals, whereas the expression of genes coding for MAPKs was unchanged, such as that in the liver. Gene expressions were also modified by ER in visceral fat tissue. However, it is impossible to determine whether the changes in mRNA observed are responsible for the prevention of diabetes in the energy-restricted group or if the changes are a consequence of the prevention.

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found, in the online version, at http://www.mdl.dk/publications_sup.htm.

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