Original Article

Low dose streptozotocin (STZ) combined with high energy intake can effectively induce type 2 diabetes through altering the related gene expression

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High energy-intake is a major factor revolved in type 2 diabetes. A number of animal models have been adopted for studying the type 2 diabetes, but they differ greatly from human type 2 diabetes. The objectives of the present study are to set up a suitable animal model, which is similar to the human type 2 diabetes, and then to understand possible molecular mechanisms underlying type 2 diabetes. Twenty five-week-old Wistar male rats were randomized into four groups. One group was fed with basal diet (BD) whereas the others consumed high-energy diet (HD) of 20% sucrose and 10% lard. Four weeks later, BD and one of HD were sampled. Other groups continued to consume HD, but one of them was treated by one injection of streptozotocin (STZ) (30mg/kg body weight). After another four weeks, all were sacrificed. Changes in body weight were recorded, and levels of glucose, TG, TC, LDL in serum were analyzed by standard methods. Moreover, expressions of genes related to energy metabolism in liver, muscle and fat were measured by real-time RT-PCR. HD had no notable differentiation with BD on bodyweight and serum indices, but it altered gene expressions in a tissue-specific manner. Two receptors of adiponectin, leptin, PPARγ, UCP2 mRNA levels in fat were up regulated, whereas most of them were down regulated in liver. STZ treatment induced symptoms of diabetes, and the gene expression mentioned above exhibited changes in both tissue- and gene-specific manners. The results suggest that a combination of low dose STZ and high-energy intake can effectively induce type 2 diabetes by altering the related gene expressions in major metabolic tissues.

Key Words: type 2 diabetes, high-energy diet, gene expression, streptozotocin, animal model

Introduction

Type 2 diabetes mellitus is an increasingly common disorder of carbohydrate and lipid metabolism.[1] There are two important characteristics of this disease, one is insulin resistance, which means the failure of peripheral tissues such as liver, muscle and adipose tissue to respond to physiologic doses of insulin, and the other one is dysfunction of pancreatic beta cell to properly secrete insulin in response to elevated blood glucose level. Insulin resistance always occurs in the early stage of type 2 diabetes, after a long time insulin resistance, a further decline of beta cells is induced, resulting in hyperglycemia and lipid metabolism confusion. It tightly linked with behavioural factors such as dietary habits and physical inactivity.[2] Studies show that the high-energy feeding can induce syndromes of glucose intolerance or insulin resistance in several species.[3]

Till now many kinds of animal model are used for researching diabetes, but neither genetic nor chemically induced animal model simulate human type 2 diabetes mellitus. Recent studies have shown that lots of genes are involved in insulin resistance and hyperglycemia, whereas molecular mechanism underlying type 2 diabetes is still not completely clear. Chemical medicine like streptozotocin (STZ) is often used for preparing type 1 diabetes animal model because of its differential wreck action to beta cells of pancreas, and interestingly, the degree of diabetes is positively related to the dose of STZ being used.[4] Furthermore, STZ can also be used for preparing non-insulin dependent diabetes animal model, such as neoonatal-strepotozotoxin rats (n-STZ rats) which were considered as a suitable model of type 2 diabetes than others,[5] but it still differs greatly from human type 2 diabetes.

Beside the gene background, unhealthy life style such as high-energy intake is correlated with type 2 diabetes. Thus, scientists try to obtain a type 2 diabetes animal model following the real course. The general strategy is using high-energy diet feeding for a period with the purpose to induce mild insulin resistance at first, and then an injection of a low dose of STZ to make partial dysfunction of beta cell for suppressing the insulin secretion, which works as a compensation to insulin resistance with the result of persistent hyperglycemia.

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However, little has been done so far to recognize the related gene expression change of this kind of diabetes animal model.

Method

Animal

Male Wistar rats with a mean body weight of about 100 g were purchased from China National Laboratory Animal Resource Center (Shanghai, China). They were kept under our animal facilities (22±1℃). Water was available *ad libitum*, with a 12-h light-dark cycle beginning at 8:00 a.m. During experiments, food was offered in daylight time. Prior to the beginning of the experiment, all rats were fed with basal diet at least for one week. The composition of basal diet was described as previously,[6] and the mineral mix and the vitamin mix were prepared according to AIN-76.[7] Body weights of rats were recorded every week. All experiments were performed under the guidelines of The National Research Council's guide for the care and use of laboratory animals and the Animal Usage Committee of The Zhejiang University of Technology.

Experimental design

To determine if the high-energy diet feeding can induce insulin resistance of rats, ten rats were randomly divided into two groups equally. One group was fed by high-energy diet, which was prepared by adding 20% sucrose (w/w) and 10% lard (w/w) into BD, for 4 weeks and described as HD in the following text, whereas the other one continued to consume BD for the same period serving as a control group (BD). Then all the ten rats were sacrificed after anaesthetized by pen-barbital. Blood samples were collected, and sera were separated and stored at -20℃ for use. Liver, peritoneal fat tissue and skeletal muscle were separated and kept at –80℃ until use.

To determine whether the low dose of STZ can induce type 2 diabetes after 4-week feeding of the high-energy diet, another independent experiment was also carried out. As described above, ten rats were equally divided into two groups and they continued to be fed with the high-energy diet for the remaining experimental period. At the same time, one group was treated with STZ (Sigma, Germany) in a dose of 30 mg/kg body weight just for 1 time, which was described as HD+STZ30 group, while the other group was regarded as the control group (HD+STZ0), which just was injected with physiological saline.[8] After another 4 weeks of high-energy feeding, all rats were killed; their sera and organ samples were collected as described above.

Measurement of lipids and serum glucose

The total cholesterol (TC), triglyceride (TG), low density lipoprotein (LDL), and glucose concentration in sera were determined by auto-biochemical analysis system (ABBOTT, ACHTECTION C8000, America) using the commercial kits (Whitman Biotech Co., Ltd, Nanjing, China) based on a modification of the cholesterol oxidize method, the lipase-glycerol phosphate oxidize method, direct method and HK method, respectively.

Gene expression analysis

Total RNA was isolated from rat tissues with TRIzol reagent (Invitrogen, USA) according to the manufacturer's protocol. cDNA was synthesized by using M-MLV reverse transcriptase kits (Takara Biochemicals, China), and a portion of 0.5 μL RT products was used directly for real-time polymerase chain reaction (PCR).[9] Primers used to amplify each gene were shown in Table 1. GAPDH transcript as a house-keeping gene was used to standardize the results by eliminating variations in mRNA and cDNA quantity and quality, and each mRNA level was expressed as its ratio to GAPDH mRNA. For the mathematical analysis, it was necessary for each transcript to determine its Ct value, the cycle number at which a fluorescent signal rises statistically above the background. The relative quantification of gene expression among the treatment groups was analyzed by the $2^{-\Delta\Delta Ct}$ method.[10]

Statistic analysis

Data were presented as mean ± SE and were analyzed by Student's t test and ANOVA using StatView 5.0 program (SAS Institute Inc., Cary, NC, USA). Values were considered statistically significant when p values were less than 0.05 or 0.01.

Results

To find out whether or not the high-energy intake can result in insulin resistance, serum glucose, TC, TG, LDL concentrations were detected in sera of rats fed with the high-energy diet for 4 weeks. As shown in Table 2, 4-week consumption of the high-energy diet did not affect the biomarkers in sera as well as the body weights.

Though there was no symptom of insulin resistance occurred, the genes expression had been changed already

Table 1. Primers used in real-time PCR analysis with SYBR Green

Gene product	Forward primer	Reverse primer
GAPDH	GACAACTTTGGCATCGTGGA	AGGCAGGGATGATGTTCTGG
Adiponectin	GGAAACTTGTGCAGGTTGGATG	GGGTCACCCTTAGGACCAAGAA
Leptin	TTCAAGCTGTGCCTATCCACAAAG	TGAAGCCCGGGAATGAAGTC
Adiponectin cerptor1(ADIPOR2)	CACAGAAACTGGCAACATCTGGA	CTGAATGACAGTAGACGGTGTGGAA
Adiponectin cerptor2(ADIPOR2)	GAAGGTCGATGGCGAGTGA	CAATGGCATTTCGGGCAAC
PPAR	TGTGGTTTCAGAAGTGCCTTG	TTCAGCTGGTCGATATCACTGGAG
Uncoupling protein 2 (UCP2)	CAGAGCACTGTCGAAGCCTACAAG	CAATGGCATTTCGGGCAAC

Table 2. Biochemical parameters in BD and HD group

	BD（n=5）	HD (n=5)
Body weight (g)	222.1±11.0	217.1±11.3
Serum glucose (mmol/L)	5.16±0.86	5.90±0.54
TG (mmol/L)	0.38±0.08	0.42±0.10
TC (mmol/L)	1.47±0.29	1.49±0.29
LDL (mmol/L)	0.52±0.11	0.56±0.12

n, number of rats; * $p<0.05$, ** $p<0.01$.

Table 3. Biochemical parameters for HD+STZ30 and HD+STZ0 group

	HD+STZ0	HD+STZ30
Body weight(g)	298.5±17.0	188.7±20.4**
Serum glucose (mmol/L)	7.03±0.94	29.44±5.36**
TG (mmol/L)	1.25±0.12	0.63±0.26**
TC (mmol/L)	1.17±0.13	2.47±0.61**
LDL(mmol/L)	0.41±0.03	0.62±0.14*

n, number of rats, * $p<0.05$, ** $p<0.01$.

because of energy over-taking. As shown in Figure 1A, in fat tissue, both leptin and adiponectin mRNA expression levels increased almost 2 to 3 folds as compared with those of BD group, respectively. ADIPOR1 and ADIPOR2 mRNA expression levels were also detected in liver, fat and skeletal muscle tissues in both HD and BD groups (Fig 1B, Fig 1C, Fig 1D). In liver both ADIPOR1 and ADIPOR2 mRNAs were decreased by the feeding of the high-energy diet. In contrast, the ADIPOR1 mRNA was 4 times higher, and ADIPOR2 mRNA exhibited a prominent increasing tendency in fat tissue of HD group than BD group. Meanwhile, no notable difference was observed in skeletal muscle between the two groups. PPARγ mRNA in fat of HD group was about 3.2-fold higher than that of BD group ($p<0.05$) (Fig 1C), but no distinct change was observed in liver and skeletal muscle (data was not shown). Moreover, a significant decrease of UCP2 mRNA was observed in liver ($p<0.05$), while a great increase was detected in fat tissue of HD group ($p<0.01$) (Fig 1B), as well as in skeletal muscle (Fig 1D).

As shown in Table 3, STZ-treatment could dramatically increase serum glucose level (HD+STZ0, 7.09 mmol/l vs HD+STZ30, 29.44 mmol/l, $p<0.01$). Similarly, serum LDL and TC level were also elevated by STZ injection (LDL: HD+STZ0, 0.41 mmol/l vs HD+STZ30, 0.62 mmol/l, $p<0.05$; TC: HD+STZ0, 2.47 mmol/l vs HD+STZ30, 1.17 mmol/l, $p<0.01$). Interestingly, serum TG level decreased in HD+STZ30 group, being almost 2 times lower than that of HD+STZ0 group (1.25 mmol/l of HD+STZ0 vs 0.63 mmol/l of HD+STZ30, $p<0.01$).

Once a low dose of STZ injection easily induced rats to get hyperglycemia and lipid level change in serum as de scribed above. In order to affirm whether the biochemical

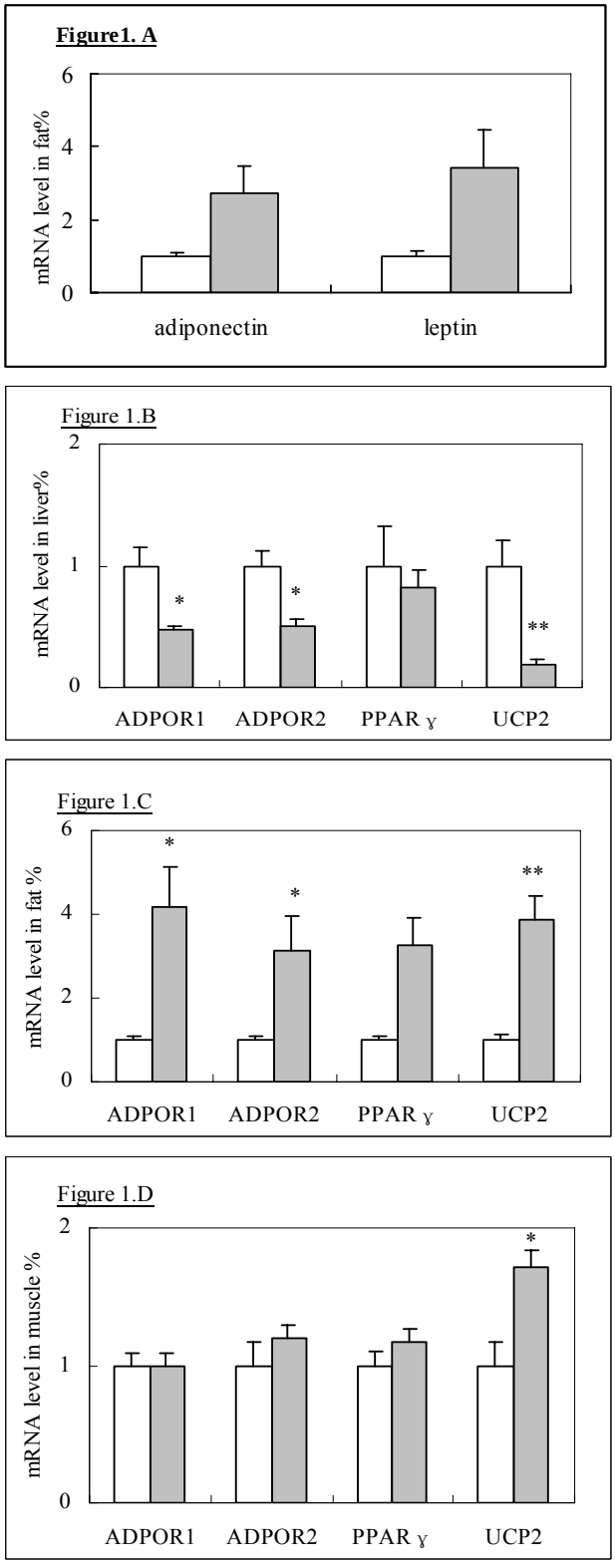

Figure 1. Gene's transcription change in liver, fat and skeletal muscle after 4-week feeding of high-energy diet. mRNA levels were analyzed by relative quantitative real-time PCR using specific primers and probes. mRNA abundances were calculated as the ratio of mRNA to GAPDH mRNA level in each cDNA sample, assigning a value of 1 to the ratio in rats fed normal chow. Values represent means ±SE for 5 rats.□, BD group; ■, HD group. *$p<0.05$, **$p<0.01$. Figure 1.A shows two important adipocytokine mRNA level changes in fat tissue; Figure 1.B shows other gene expression change in liver; Figure 1.C shows corresponding gene expression in fat; Figure 1.D shows corresponding gene expression level in skeletal muscle.

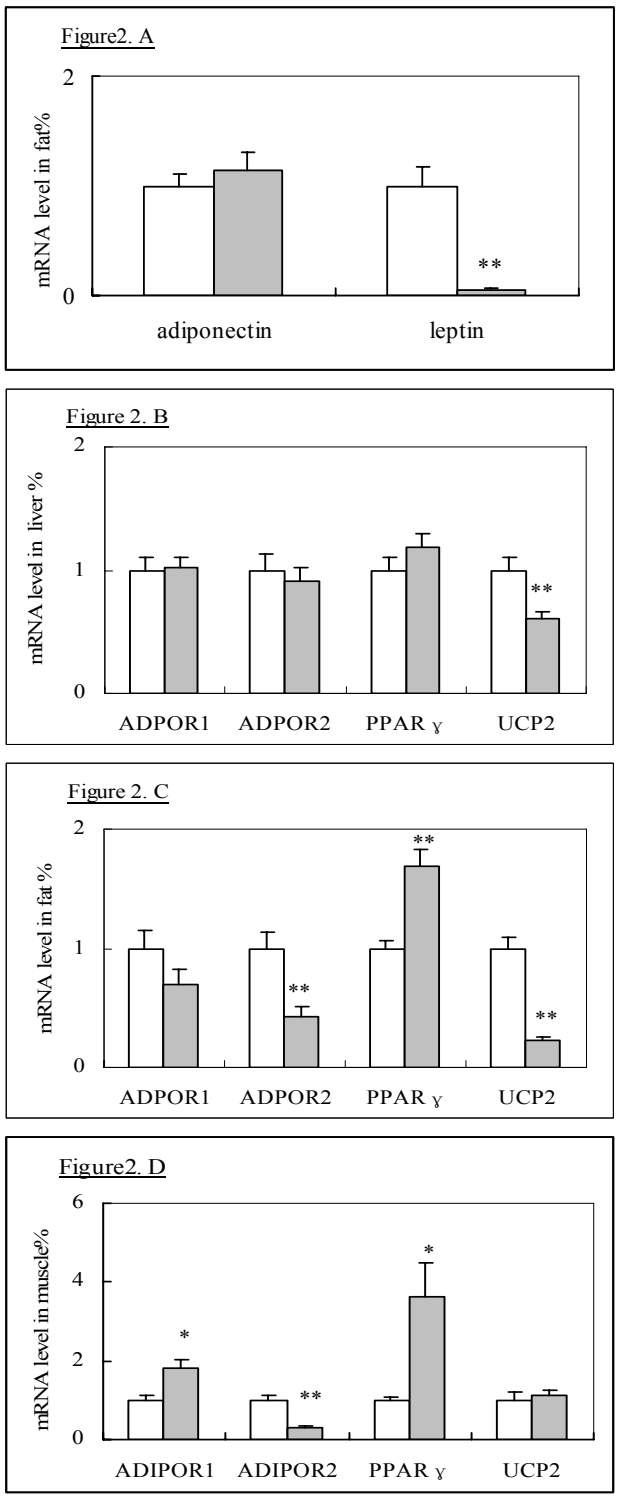

Figure 2. Gene's transcription change in liver, fat and skeletal muscle after another 4-week feeding of high-energy with a combination of a single injection of STZ. mRNA levels were analyzed by relative quantitative real-time PCR using specific primers and probes. mRNA abundances were calculated as the ratio of mRNA to GAPDH mRNA level in each cDNA sample, assigning a value of 1 to the ratio in rats fed normal chow. Values represent means ±SE for 5 rats.□, HD+STZ0 group, ■, HD+STZ30 group. *p<0.05, **p<0.01. Figure 2.A shows two important adipocytokine mRNA level changes in fat tissue; Figure 2.B shows other gene expression change in liver; Figure 2.C shows corresponding gene expression in fat ; Figure 2.D shows corresponding gene expression level in skeletal muscle.

change related to molecular regulation, we detected the gene expression levels in liver, fat and skeletal muscle of HD+STZ30 group and HD+STZ0 group. In liver, the expression of most selected genes had no notable change except that a remarkable decrease in UCP2 mRNA was observed (p<0.01) (Fig 2B). However, STZ greatly influenced the expression of some genes in fat tissue. ADIPOR2 and UCP2 mRNA levels decreased markedly (p<0.01), while PPAR ㆍmRNA level increased significantly (p<0.01) in the HD+STZ30 rats (Fig 2C).

Similarly, leptin mRNA decreased significantly (p<0.01), whereas adiponectin mRNA was not changed so much in the HD+STZ30 rat (Fig 2A). In the skeletal muscle, PPARγ (p<0.01) and ADIPOR1 (p<0.05) mRNA levels were up-regulated, while the expression of AIDPOR2 gene was down-regulated by STZ treatment (p<0.01) (Fig 2D).

Discussion

In the present study we found that the high-energy feeding did not show a great effect on the body weight as well as biochemical parameters of sera. But it had induced the expression level of the genes that are tightly involved in energy metabolism in major metabolism regulation tissue. This molecular modulation may rebalance the energy metabolism in a new level, and result in the stabilization of blood glucose level and all other lipid indices.

Insulin resistance is one of the most important risk factors associated with diabetes, especially for type 2 diabetes. Recent studies have provided evidence that adipose tissue may play a crucial role in the development of insulin resistance and type 2 diabetes through the secretion of a variety of biologically active molecules (adipocytokine). The most important two kinds of such cytokines are adiponectin and leptin.[11,12,13] Adiponectin is a member of the adipocytokine family that exclusively expresses in differentiated adipocytes and plays an important role in regulating energy homeostasis, including the glucose and lipid metabolism associated with increased insulin sensitivity.[14] Leptin, an adipocyte-derived hormone, functions as the afferent signal in a feedback loop regulating adipose tissue mass,[15] with its major site of action in hypothalamus,[16] and can cause both blood glucose and insulin levels decreasing.[17] In the present study, the feeding of the high-energy diet caused a great increase in the mRNA levels of adiponectin and leptin genes, indicating that the adipogenesis and lipolysis are working simultaneously to regulate the energy balance. In one hand, the animal enhances the lipid storage ability in fat; meanwhile in other hand body also enhances utilization ability of lipid in fat. This might explain why high-energy feeding didn't stimulate rats to get more body weight gain, and also suggested that the high-energy intake enhanced the metabolic ratio in a new level.

ADIPOR1 and ADIPOR2 are newly identified receptors for adiponectin. ADIPOR1 is ubiquitously expressed with highest levels in the skeletal muscle, while ADIPOR2 is predominantly expressed in the skeletal muscle and liver.[18] Recent research showed that activation of AMP-activated protein kinase and PPARγ by adiponectin was mediated by ADIPOR1 and ADIPOR2, and mRNA levels of both receptors were positively correlated with glucose disposal,[19] suggesting that the down-regulation or altered function of ADIPOR1 and ADIPOR2 may be responsible for development of insulin resistance in peripheral tissue, and may

contribute to increase susceptibility in type 2 diabetes. High-energy feeding increased and decreased ADIPOR1and ADIPOR2 mRNA levels in fat and liver, respectively, indicating that the prominent organ to deposite lipid has been changed from liver to fat. The elevation of PPARγ mRNA levels in the fat in the present study also support the hypothesis described above. PPARγ is a ligand-activated transcription factor and belongs to the nuclear hormone receptor superfamily.[20] It is originally identified as a crucial factor in adipogenesis and glucose metabolism.[21, 22]

Uncoupling proteins (UCPs) are mitochondria transporters present in the inner membrane of mitochondria.[23] UCP2 is expressed in adipose tissue, skeletal muscle, and macrophages to participate in intermediary metabolism, particularly in fatty acid metabolism.[24] The function of UCP2 may export fatty acid outside of mitochondrial matix when a large excess of fatty acids present in the mitochondrial matix.[25] Therefore, the up-regulation of UCP2 mRNA level in fat and the down-regulation in liver by the feeding of the high energy diet observed in the present study might add an evidence to the above presume.

A low dose of STZ injection (30mg/kg body weight) after 4-week feeding of the high-energy diet has shown a great effect to induce diabetes by markedly elevating serum glucose, total cholesterol, and LDL levels. This combination of the STZ treatment with the high-energy feeding caused serum glucose level higher than a single injection of STZ with a dose of 55mg/kg, even much higher than that of 65mg/kg.[26] Recently, more and more researchers have paid attention to produce diabetes rats by such treatment. For example, Zhang et al. induced type 2 diabetes successfully by using 4 months old rats fed high-fat diet for 2 months and treated with STZ (15mg/kg body weight).[27] And Reed et al. did a similar experiment adopting a 2-week-feeding of a high-fat diet and an injection of STZ 50mg/kg.[28] Just as reported in previous research, the serum symptoms induced by this treatment adopted in the present study were more similar to those of type 2 diabetes than those of type 1 diabetes. In addition, when the serum biochemical parameters obtained from 1 week and 4 weeks after STZ treatment were compared, it was found that they became worse when rats continued to take the high-energy diet (data not shown). This phenomenon may be explained as follow. STZ injection barely wrecked part of the beta cell in islet, and the subsequent high-energy feeding induced the insulin resistance and caused further disfunction of beta cell, at last made symptoms more serious.

The gene expression of such diabetes model scarcely had been studied yet. In the present study, some genes possibly related to the type 2 diabetes were assayed in different tissues. In fat, a notable decrease in ADIPOR2 mRNA induced by the combination of the high-energy diet feeding and the STZ-injection might indicate that a restrain of lipid synthesis and storage occured with an accompany of type 2 diabetes, which may explain why a great loss of body weight was observed in HD+STZ30 group, and it may also indicate that insulin desensitivity occurred in the fat. In addition, STZ injection affected the expression of ADIPOR2 gene more greatly than that of ADIPOR1, indicating that ADIPOR2 may play a more important role in modulating lipid metabolism in the fat. Moreover, the down-regulation of leptin and UCP2 gene expression observed in the STZ-treated fat may account for the lose of fat tissue, since both leptin and UCP2 transcription levels had positive mutuality to the mass of adipocytes and FFA concentration in this tissue.[29] The down-regulation of UCP2 gene expression may increase the risk for cells to be damaged by ROS. In contrast, the significant increase in PPARγ mRNA level in STZ-induced diabetes may protect the fat tissue from declining sharply, because PPARγ functions as an important coregulator of lipid homeostasis and a key regulator of adipocyte differentiation and lipid storage.[30]

The genes transcription in skeletal muscle was also influenced greatly by STZ-treatment. The expression of ADIPOR1 and ADIPOR2 mRNAs changed in an opposite direction with an elevation of ADIPOR1 and a decline of ADIPOR2 mRNA levels. These results indicated that ADIPOR2 expression in skeletal muscle may be immediately inhibited by STZ and the up-regulation of ADIPOR1 mRNA may serve as a compensation to keep cell capacity to intake and utilize glucose and fatty acid, and thus mitigate muscle cells from desensitization to insulin. The raise of PPARγ mRNA level hinted that the maintenance of lipid function still existed in skeletal muscle.

In conclusion, the type 2 diabetes rats obtained from the method adopted in the present study possessed representative symptoms of human type 2 diabetes according to common hyperglycemia and lipid metabolism disorder. Thus, high-energy feeding combined with a low dose of STZ injection was a practical method to obtain type 2 diabetes model, it could induce diabetes efficiently. The present study focused on the molecular regulation of the type 2 diabetes model, and found that the changes in the gene expression are mostly consistent to that reported in human type 2 diabetes.

Acknowledgements

This work was supported by grants from Zhejiang University of Technology and grants from Zhejiang Province to ZW Fu (No. 2004C14003).

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