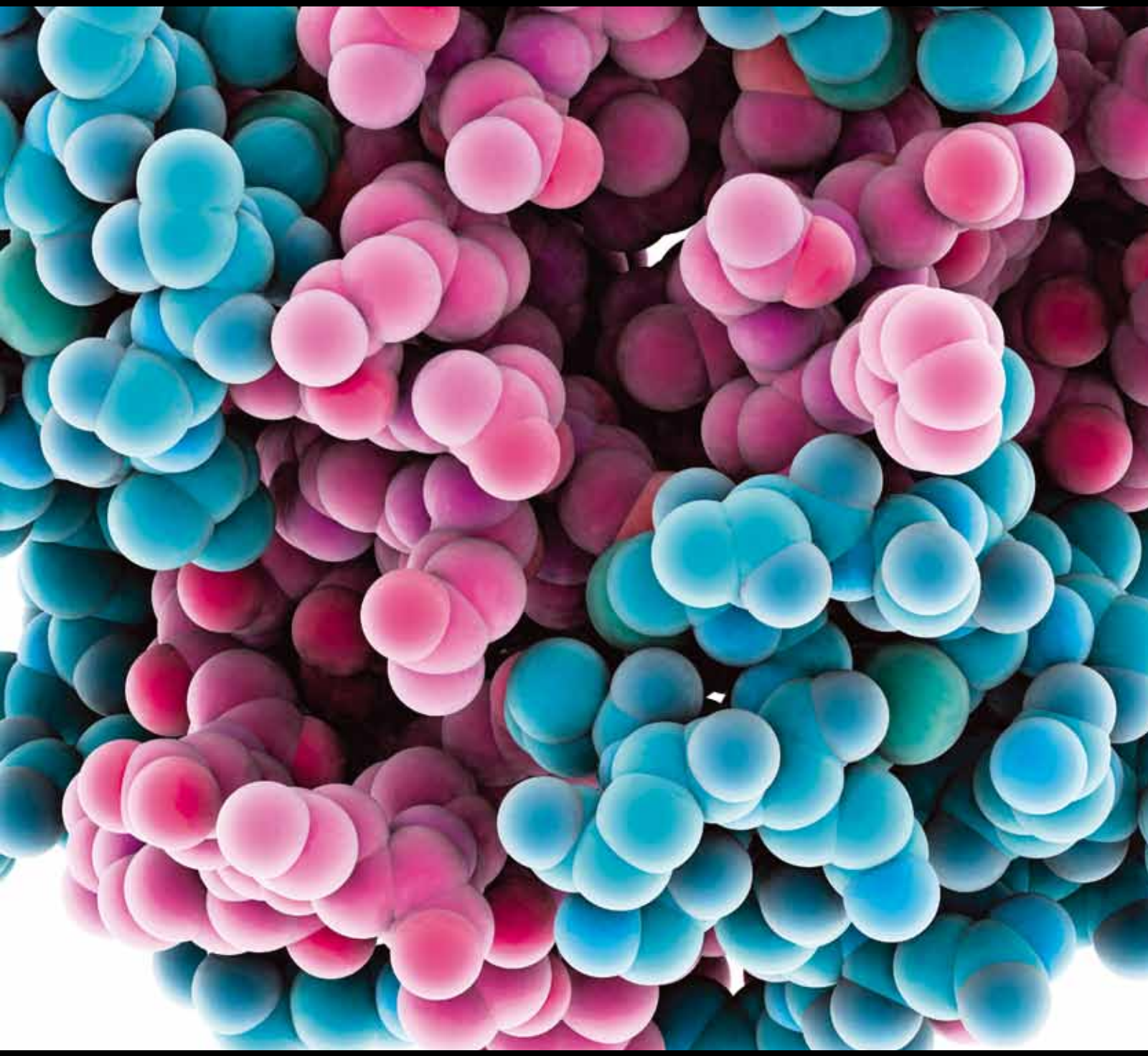
Journal of Diabetes Research

Cytokines and Diabetes Research

Guest Editors: Jian Xiao, Ji Li, Lu Cai, Subrata Chakrabarti, and Xiaokun Li



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Editorial Board

Contents

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Editorial

Cytokines and Diabetes Research

Jian Xiao,[1] Ji Li,[2] Lu Cai,[3] Subrata Chakrabarti,[4] and Xiaokun Li[1]

[1] *School of Pharmacy, Wenzhou Medical College, Wenzhou 325035, China*
[2] *School of Medicine and Biomedical Sciences, University at Buffalo-SUNY, Buffalo, NY 14214, USA*
[3] *School of Medicine, University of Louisville, Louisville, KY 40202, USA*
[4] *London Health Science Center Pathology, University of Western Ontario, London, ON, Canada N6A 5A5*

Correspondence should be addressed to Xiaokun Li; xiaokunli@163.net

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In recent years, the role of the inflammatory system in the pathogenesis of diabetes has been increasingly investigated. Cytokines, a group of proteins that are expressed by several cell types, act as immune mediators and regulators. Depending on the period of pregnancy, a predominant inflammatory profile is defined by increased production of cytokines. Insulin resistance has been associated with abnormal secretion of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and Interleukin-6 (IL-6) and decreased production of anti-inflammatory mediators such as IL-4 and IL-10. Despite some controversies regarding specific cytokine levels, type 2 diabetes mellitus (T2DM) is currently regarded as a chronic inflammatory disease, while type 1 diabetes (T1D) is considered to be a T-helper-(Th)-1 autoimmune disease.

Extensive research in animals and in humans over the last decade has revealed important functions of cytokines in diabetes; adiponectin (APN) and leptin can decrease hepatic gluconeogenesis, resistin (REN) can increase hepatic gluconeogenesis and glycogenolysis, IL-6 can decrease glycogen synthesis, and TNF-α can decrease glucose uptake in liver. Both of them can block hepatic insulin signalling by interfection of insulin receptor signalling and insulin signal transduction. Thus, cytokines are involved in nearly every facet of immunity, inflammation, and development of diabetes.

In this special issue, we have invited some papers hoping to shed light on some aspects of this very interesting field. We have collected 7 papers by scientists from 5 countries. In the submitted research papers, Y. Li et al. summarize recent findings regarding the relationship between adipocytokines and hepatic insulin resistance. Excessive adipose tissue may be detrimental partially through secretion of the following cytokines: TNF-α, IL-6, and resistin. In contrast, the presence of adipose tissues is vital in the prevention of hepatic insulin resistance via secretion of the following cytokines: leptin and adiponectin. While J. Su and colleagues review the relationship between the endoplasmic reticulum (ER) and autophagy, inflammation, and apoptosis in DM to better understand the molecular mechanisms of diabetes, the authors suggest that the ER is therefore an attractive potential therapeutic target, and maintaining or improving ER function appropriately may prevent diabetes. Z. Meng et al. concluded that ethanol causes glucose intolerance by increasing hepatic expression of 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) and glucocorticoid receptor (GR), which leads to increased expression of gluconeogenic and glycogenolytic enzymes. In the following papers, J. Liu et al. have shown that uncoupling proteins (UCPs) may affect the development of DM through decreasing mitochondrial membrane potential, increasing energy expenditure especially through glucose and lipid metabolisms, downregulating ROS generation, and gene polymorphisms. In a very interesting research paper, J. Vcelakova et al. have shown, that in T1D patients, important immune response-related pathways were involved. These important immune response-related processes largely included the induction of Th17 and Th22 responses, as well as cytoskeletal rearrangements, MHCII presentation, and the upregulation of CD4, TGF-beta, and STAT3. These findings potentially suggest that these processes could be utilised as predictive markers for the development of T1D or as molecular targets for the repression of specific immunocompetent

cell populations for the treatment of diabetes. On the other hand, H. Meng and colleagues demonstrate that amyloid precursor protein 17 peptide (APP17 peptide) has a comprehensive therapeutic effect on diabetic encephalopathy, particularly through improving glycol metabolism. Finally, M. Cui et al. have shown that AMPK activation, which was represented by the level of p-AMPK, did not correlate with the improvement of metabolic conditions in diabetes mice, implying that AMPK activation may not participate in mediating the beneficial effects of chronic caloric restriction (CR) or exercise. However, the autophagy activity might be related to the improved metabolic conditions; thus autophagy may play a role in mediating the effects of chronic CR.

Acknowledgment

Finally, we would like to thank all contributors to this special issue for their participation. We hope that this special issue will be helpful for the cytokine research and the development of new therapeutic drugs for the modern treatment of diabetes and diabetes complication.

Jian Xiao
Ji Li
Lu Cai
Subrata Chakrabarti
Xiaokun Li

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Research Article

Effects of Amyloid Precursor Protein 17 Peptide on the Protection of Diabetic Encephalopathy and Improvement of Glycol Metabolism in the Diabetic Rat

Heng Meng,[1,2] Duo Zhang,[2] and Haishan Yang[1]

[1] *Department of Radiology, China-Japan Union Hospital of Jilin University, Changchun 130033, China*
[2] *Department of Radiology, Affiliated Hospital of Beihua University, Jilin 132011, China*

Correspondence should be addressed to Haishan Yang; ibiocc0431@gmail.com

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Researchers have proposed that amyloid precursor protein 17 peptide (APP17 peptide), an active fragment of amyloid precursor protein (APP) in the nervous system, has therapeutic effects on neurodegeneration. Diabetic encephalopathy (DE) is a neurological disease caused by diabetes. Here we use multiple experimental approaches to investigate the effect of APP17 peptide on changes in learning behavior and glycol metabolism in rats. It was found that rats with DE treated by APP17 peptide showed reversed behavioral alternation. The [^{18}F]-FDG-PET images and other results all showed that the APP17 peptide could promote glucose metabolism in the brain of the DE rat model. Meanwhile, the insulin signaling was markedly increased as shown by increased phosphorylation of Akt and enhanced GLUT4 activation. Compared with the DE group, the activities of SOD, GSH-Px, and CAT in the rat hippocampal gyrus were increased, while MDA decreased markedly in the DE + APP17 peptide group. No amyloid plaques in the cortex and the hippocampus were detected in either group, indicating that the experimental animals in the current study were not suffering from Alzheimer's disease. These results indicate that APP17 peptide could be used to treat DE effectively.

1. Introduction

Diabetes mellitus, or simply diabetes, is a group of metabolic diseases in which a person has high blood sugar, either because the pancreas does not produce enough insulin or because cells do not respond to the insulin that is produced [1–3]. Insulin causes cells in the liver, skeletal muscles, and fat tissue to absorb glucose from the blood. In the liver and skeletal muscles, glucose is stored as glycogen, and in fat cells (adipocytes) it is stored as triglycerides [4, 5]. Diabetes can be divided into 3 main types: Type 1 diabetes, which results from the inability to produce insulin; Type 2 diabetes, resulting from insulin resistance; and gestational diabetes [6–8]. Type 2 diabetes, without proper treatment, can cause many complications, including hypoglycemia, diabetic ketoacidosis, and nonketotic hyperosmolar coma [9]. Adequate treatment of diabetes is consequently vital. Diabetic encephalopathy (DE) is caused by diabetes [10]. The complications of DE include memory loss, dementia, coma, seizures, and finally death. The defects in patients include lethargy, poor judgment and coordination of limbs, dementia, and muscle twitching [11].

Amyloid precursor protein (APP) is a transmembrane protein with six isoforms in the central nervous system (CNS), of which APP-695 is the most important [12, 13]. Hydrolysis of the N-terminus of APP-695 by α-secretase yields a soluble 100 kDa fragment, sAPPα, which has a unique function of promoting neuronal growth [14, 15]. The active domain responsible for this activity has been identified in the 319–335 peptide segment of APP-695 and is known as APP17 peptide. Previous studies reported that APP17 peptide could promote axonal growth, increase synaptic density, and protect neurons from ischemic damage [16, 17]. In a previous report from our group, we found that APP17 peptide ameliorates hippocampal neurodegeneration in mice afflicted by streptozotocin- (STZ-) induced diabetes [18]. The APP17 peptide has also showed neuroprotective effects through activation of specific signal transduction pathways associated with neuronal survival [19]. However, little is known about

the effects of APP17 peptide on encephalopathy caused by diabetes mellitus. In the present study, we use multiple experimental approaches to investigate the effect of APP17 peptide on changes in learning behavior and glycometabolism in rats.

2. Materials and Methods

2.1. Experimental Animals and Creation of Animal Model. Male Wistar rats (weighing 180–200 g) were supplied by the Laboratory Animal Center of Beijing. All animal experiments were conducted according to the guidelines of the local animal use and care committees and executed according to the National Animal Law. The animals were divided into three groups: normal controls (CON, $n = 25$), diabetic (DE, $n = 25$), and APP17 peptide-protected group (DE + APP17 peptide, $n = 25$). APP17 peptide was synthesized by solid phase method and purified in the authors' laboratory. Rats in the DE + APP17 peptide group were given APP17 peptide for 4 weeks after STZ (Sigma) treatment (0.7 μg per rat, s.c. daily). STZ was prepared before each use at 20 mg/mL in 0.1 M pH 4.4 citrate buffer and was injected at 150 mg/kg, i.p., into rats which had been fasted for 12 h prior to receiving the injection. Four days later, nonfasting blood glucose in a tail-vein sample was determined by a glucose analyzer; a value >15 mM/L was accepted as a successfully created diabetic model.

2.2. Learning and Memory. Morris water maze tests were performed after training for 12 weeks. After the rats were familiar with the testing environment, normal training was performed from the second day. Orientation test: rats were trained twice per day, one time in the morning and one time in the afternoon. Each training session lasted for 120 sec, and the gap time was 30 s. The training lasted for 4 days. The starting area was randomly selected, and the number of times rats touched the platform in 120 sec was recorded. The platform was removed, and the rats were placed into water at the opposite side of the platform. The percent of residence time in the center area and number of times of passing the former platform in 120 sec were recorded.

2.3. In Vivo Positron Emission Tomography (PET) Scans. PET studies were performed on the rats suffering from diabetes or DE ($n = 20$ per group). The PET protocol was the following: animals were anesthetized by isoflurane (3% for induction, 1%–1.5% for maintenance), and the respiration rate was monitored during the experiment. The body temperature of the animals was maintained at 37°C throughout the PET examination using a warming system. PET images were recorded on a high-resolution small-animal PET imaging device with a spatial resolution of 1.35 mm and a field of view (FOV) of 7.6 cm (MicroPET Focus 220, Siemens Medical Solutions, Inc., Hoffman Estates, IL, USA) [20]. The mice were scanned with an energy window of 350–650 keV and a coincidence time window of 6 sec. Brain emission scans were acquired in 3D mode during 60 min after a tail-vein bolus injection MBq of [^{18}F]-FDG (CisBio, Orsay, France).

Blood glucose concentration was measured once during the scan using a One Touch Ultra Glucose Meter (LifeScan, Issy-Les-Moulineaux, France). Blood glucose concentrations were in the normal range, and no difference was detected between the various genotypes or ages of the groups (2-way analysis of variance (ANOVA) with genotype and age as between-subject factors, Fs (<1 for the main factors)). The PET images were reconstructed with the 2D iterative ordered-subset expectation maximization (FORE 2D OSEM) mode. Sixteen subsets and 4 iterations were used for reconstruction. The mean [^{18}F]-FDG activities, corrected for radioactive decay, were evaluated for each VOI on integrated PET images recorded during a 30–60 min acquisition period. Standardized uptake values (SUVs) were obtained for each VOI by dividing the mean [^{18}F]-FDG activities by the injected dose and the animal weight.

2.4. Biochemistry Markers. The brains of rats in each group after the test of abilities of learning and memory were collected on the ice, and then the hippocampus was dissected. Tissues were crushed and centrifuged at the speed of 2000 r/min for 10 min. The supernatant was collected, and the activities of SOD, GSH-Px, and CAT and content of MDA in the rat's hippocampal gyrus were investigated. Coomassie brilliant blue staining was used to detect protein concentration.

2.5. Harris Hematoxylin and Eosin (H&E) Staining. Thirty micron (μm) brain coronal sections were collected from every 200 μm section. The sections were deparaffinized, with two changes of xylene, 10 min each. The sections were rehydrated in 2 changes of absolute alcohol for 5 min each, 95% alcohol for 2 min, and 70% alcohol for 2 min, washed briefly in dH_2O, and stained in Harris hematoxylin solution for 8 min. The sections were washed in running tap water for 5 min and differentiated in 1% acid alcohol for 30 sec. The slides were then washed in running tap water for 1 min and stained in 0.2% ammonia water or saturated lithium carbonate solution for 30 to 60 sec. The slides were then washed in running tap water for 5 min, rinsed (10 dips) in 95% alcohol, and counterstained in eosin-phloxine solution for 30 sec. The slides were dehydrated in 95% alcohol, 2 changes of absolute alcohol, 5 min each. The slides were cleaned in 2 changes of xylene, 5 min each, and mounted with xylene-based mounting medium. The neurons in CA1 in the hippocampus were observed using an optical microscope.

2.6. IHC Staining Test. After dissecting tissues at 5 μm and fixed in 4% paraformaldehyde for 10 min, slides were incubated 2 to 3 times in xylene for 10 min each and then incubated twice in 100% ethanol for 2 min each. The slides were hydrated in 95%, 70%, 50%, and 30% ethanol for 2 min each. Slides were placed into buffer containing 5% normal goat serum for 10 min. Slides were incubated in a humidified chamber overnight with primary antibody (rabbit anti-rat Akt/PKB 1 : 500, rabbit anti-rat GLUT4 1 : 1000). They were washed in 5 m in buffer for 3 times and incubated with secondary antibody in a humidified chamber for 30 min. DAB and hematoxylin staining, 5 discontinue brain sections were

selected, and 5 fields were selected randomly. The numbers of Akt/PKB and GLUT4 positive cells in CA1 were counted.

2.7. Cell Proliferation Assays. The inhibition of cell proliferation and viability of PC-12 cells was determined using the WST-1 (Roche) assay Kit. Cells were placed at 8,000 per well in 96-well plates in their respective growth medium with FBS reduced to 2%. The cells were allowed to grow for 24 h and then treated with different drugs. After 24 h, the WST-1 reagent was added to the plates according to manufacturer's protocol, and absorbance was read at 450 nm with an ELISA reader (Tecan). The results were used for calculating IC_{50} of APP17 peptide.

2.8. $[^3H]_2$-Deoxyglucose (2-DOG) Uptake Assay. PC-12 cells were placed at 8,000 per well in 96-well plates in their respective growth medium with FBS reduced to 2%. PC-12 cells were pretreated with Akt and PI-3K inhibitor 124005 (Millipore, USA) and Akt inhibitor 124011 (Millipore, USA). The cells were allowed to grow on 12-well plates and treated with APP17 peptide. The cells were serum-starved in DMEM containing 0.1% FBS for 3 h. The cells were washed twice with PBS and incubated in 0.45 mL of KRH (20 mM HEPES, pH 7.4, 136 mM NaCl, 4.7 mM KCl, 1.25 mM $MgSO_4$, and 1.25 mM $CaCl_2$) in the presence or absence of 10 nM insulin for 30 min at 37°C. For [^{3}H] 2-DOG uptake, 50 L of reaction mixture containing 5 μCi of 2-[1,2-3H]-deoxy-D-glucose (PerkinElmer Life Sciences) and 1 mM 2-DOG was added to each well for 5 min at room temperature. The reaction was stopped by the addition of 50 μL of 200 mM 2-DOG into each well. The cells were washed two times with ice-cold PBS and solubilized in 0.5 mL of 0.1% SDS at room temperature for 10 min. The cells were washed two times with ice-cold PBS and solubilized in 0.5 mL of 0.1% SDS at room temperature for 10 min. The incorporated radioactivity was determined by liquid scintillation counting of 400 μL of each sample in triplicate. Nonspecific passive [^{3}H] 2-DOG uptake control measured as a treatment with 10 μM cytochalasin B was subtracted from each value.

2.9. Western Blot. Following heating at 100°C for 5 min, 20 μg of protein was run. The *western blot* was run on an SDS-PAGE gel until the blue front was at the bottom of the gel. The gel was then transferred to a nitrocellulose membrane for 0.5 A. The membrane was blocked for 1 h in 5% skim milk in 1 × PBST. The membrane was incubated in the primary antibody (rabbit anti-rat p-Akt 1 : 500, rabbit anti-rat GLUT4 1 : 1000, rabbit anti-rat β-actin 1 : 200) at 4°C overnight. The membrane was then washed 3 times for 5 to 10 min in 50 mL of 1 × PBS with 0.1% Tween 20 at room temperature (RT). The membrane was incubated with goat anti-rabbit 1 : 200 for 1 h at RT in 1 × PBST, washed 3 × 10 min, and rinsed with dH_2O. Detection of the protein was determined by the use of the ECL kit (2 mL/membrane). Briefly, in separate tubes, the black and white ECL solutions were mixed in a 1 : 1 ratio. The solution was then aliquoted onto the membranes and left standing for 1 min. The ECL was then drained off the membrane, and the membrane was wrapped in plastic and exposed to film. The expression of protein was compared with β-actin (a positive control).

2.10. Statistical Analysis. Data were expressed as mean ± standard deviation (M ± SD). Group differences in the swimming time in the Morris water maze test and the number of errors in the passageway water maze test were analyzed by SPSS 11.0 using Windows software to conduct two-way analysis of variance (ANOVA, equal variances assumed by S-N-K) on repeated measurements. Other data were analyzed by SPSS 11.0 using Windows software to conduct one-way ANOVA (equal variances assumed by S-N-K). A post hoc test was used to obtain the P values. A $P < 0.05$ was considered significant.

3. Results and Discussion

3.1. Memory Ability. The rats of the DE group were polydipsia, polyphagia, polyuria and weight loss, yellowish color, poor spirit of the late, slow-moving symptoms. As shown in Table 1, at the beginning of generating animal model, the values of blood glucose in DE and DE + APP17 peptide groups were much higher than control group on 13th weeks ($P < 0.01$), while the body weight of mice in 3 groups remained the same ($P > 0.05$). After the treatment, the values of blood glucose in DE + APP17 peptide group were decreased, while body weight increased compared with DE group; the difference was significant ($P < 0.05$) (Figures 1(a) and 1(b)). Using the Morris water maze test, the rats treated with APP17 peptide had a prolonged swimming time ($P < 0.05$) and made significantly more errors when compared with the control group ($P < 0.05$). The rats showed reversed behavioral alternation with levels returning close to that of rats in the control group (Figure 1(c)).

3.2. The APP17 Peptide and Glucose Metabolism in the Brain of the DE Rat Model. As shown in Table 1, at the beginning of the generation of the animal model, the blood glucose in the DE and DE + APP17 peptide groups was significantly higher than that in the control group ($P < 0.01$), while the body weight of mice in 3 groups remained the same with no significant difference ($P > 0.05$). After the treatment, the blood glucose in the DE + APP17 peptide group decreased, and the body weight significantly increased compared with DE group ($P < 0.05$).

The mean [^{18}F]-FDG activities, corrected for radioactive decay, were evaluated for each VOI on integrated PET images recorded during a 30–60 minute acquisition period. Standardized uptake values (SUVs) were obtained for each VOI by dividing the mean [^{18}F]-FDG activities by the injected dose and the animal weight. Regional FDG data were normalized by the FDG uptake within the cerebellum [21]. To study glycol metabolism, changes of [^{18}F]-FDG-PET images were recorded in the DE rat model. After anatomo-functional combination, cerebral regions such as the cortex, the hippocampus, the striatum, and the cerebellum were outlined on PET images (Figure 2). A significant positive

Table 1: Blood glucose and body weight of mice in 3 groups ($x \pm s$, $n = 1$). Different letters represent the significant difference at $P < 0.05$.

Group	Blood (mmol/L)		Body weight (g)	
	0 w	13 w	0 w	13 w
CON	5.40 ± 0.41	5.56 ± 0.35	240.87 ± 5.44	350.32 ± 19.19
DE + APP17	21.73 ± 1.53**	16.43 ± 1.12**	238.97 ± 5.91	250.58 ± 15.22**
DE	24.28 ± 1.98**	22.96 ± 1.35**##	235.00 ± 12.1	180.02 ± 14.50**##

*$P < 0.05$, **$P < 0.01$ versus CON, #$P < 0.05$, ##$P < 0.01$ versus DE.

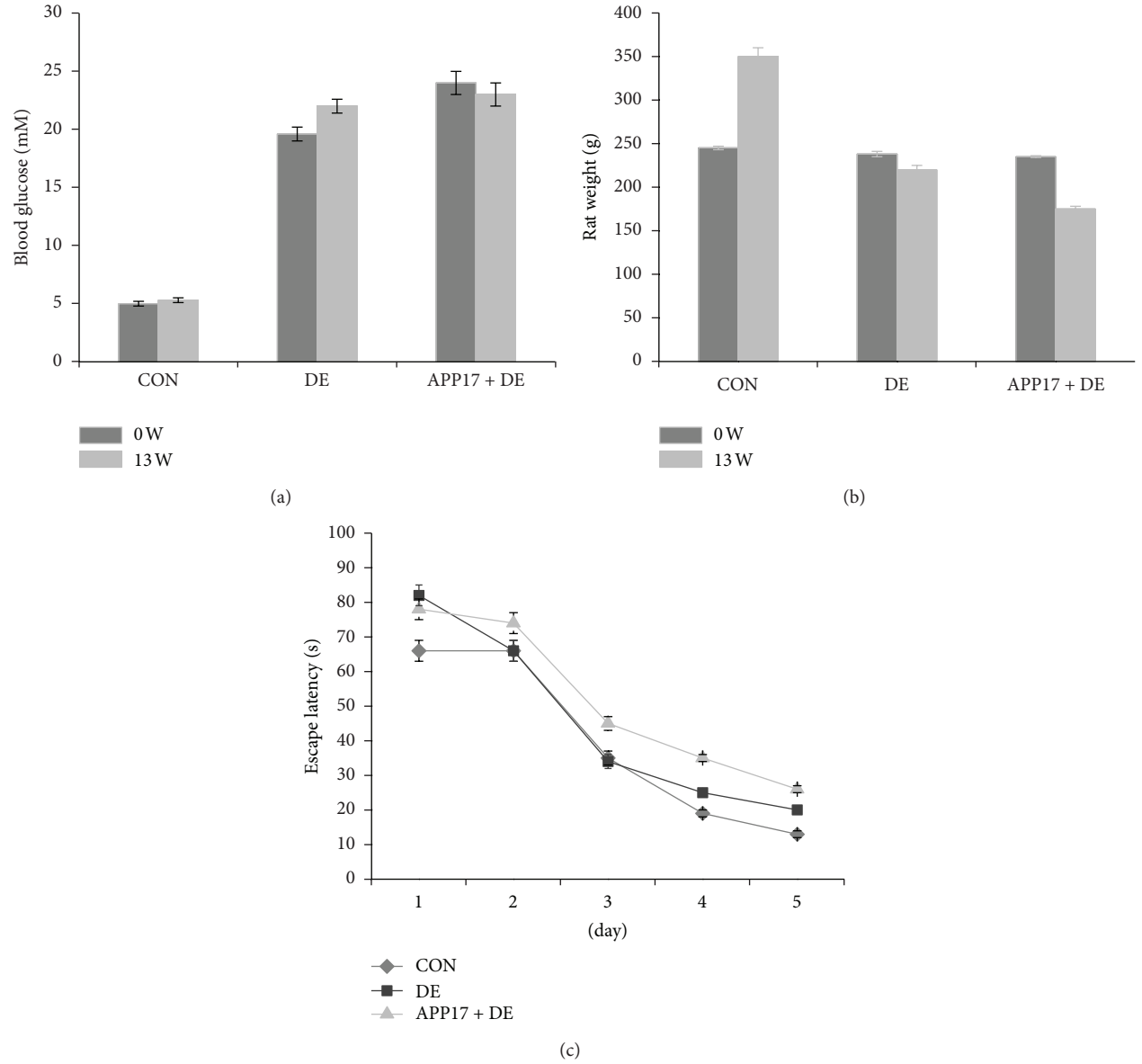

Figure 1: (a) Blood glucose and (b) body weight of mice in 3 groups. (c) The ability analysis of learning and memory of mice in 3 groups.

correlation was found between the DE group and DE + APP17 peptide group and the [^{18}F]-FDG uptake in the cortex and the hippocampus. Evaluation of glycol metabolism in animals revealed a decrease of cortical and hippocampal glucose uptake in the DE group compared with the CON group. In the DE + APP17 peptide group, the glucose uptake was increased, as compared with DE group.

3.3. Biochemistry Alterations. Compared with the control group, the activities of SOD, GSH-Px, and CAT in the rat hippocampal gyrus in the DE group decreased significantly ($P < 0.01$). Compared with the DE group, the activities of SOD, GSH-Px, and CAT in the rat hippocampal gyrus were increased, whereas the MDA decreased significantly in the DE group ($P < 0.05$ or $P < 0.01$ (Table 2)).

Table 2: Changes on biochemistry of rats in 3 groups ($x \pm s$, $n = 10$).

Group	SOD (U/mg·pro)	GSH-Px (U/mg·pro)	CAT (U/mg·pro)	MDA (nmol/mg·pro)
CON	55.48 ± 5.22	0.072 ± 0.015	6.11 ± 0.80	8.01 ± 2.19
DE	44.87 ± 10.45[#]	0.050 ± 0.011[#]	2.42 ± 0.50[#]	15.32 ± 3.44[#]
APP17 + DE	60.50 ± 8.56[#]	0.062 ± 0.005[#]	3.98 ± 0.82[#]	10.15 ± 1.76[#]

[#]The significant difference at $P < 0.05$.

Table 3: The number of positive cells of neurons in hippocampus of rats in 3 groups ($x \pm s$).

Group	Akt/PKB positive cells	Live	Death
CON	35.56 ± 4.60	10	0
DE	18.75 ± 3.13[#]	7	3
APP17 + DE	31.68 ± 5.51	9	1

[#]Significant difference at $P < 0.05$.

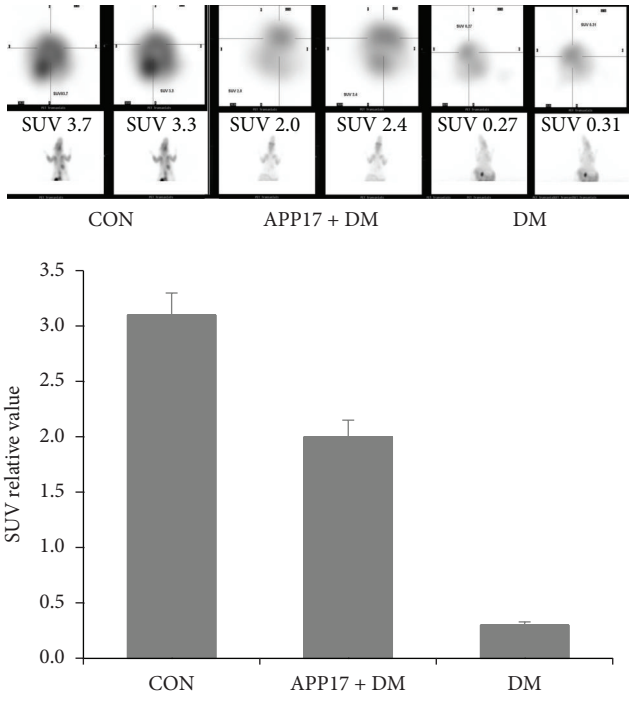

Figure 2: Evaluation of brain glycometabolism in DE animals by PET/CT.

3.4. Expression of Akt/PKB and GLUT4 after Treatment of APP17 Peptide. The results of the IHC indicated that, compared with the control group, the number of Akt/PKB positive cells in the hippocampus of the DE group was reduced. In contrast the Akt/PKB positive cells in DE + APP17 peptide group was similar to those in the control group. The numbers of Akt/PKB positive cells in hippocampus in 3 groups are shown in Table 3. In the results of western blotting, APP17 acutely stimulated Akt phosphorylation in the group of treatment, compared with control cells (Figure 3). The expression of GLUT4 in membrane was obviously decreased in the rat hippocampal gyrus in DE group ($P < 0.01$).

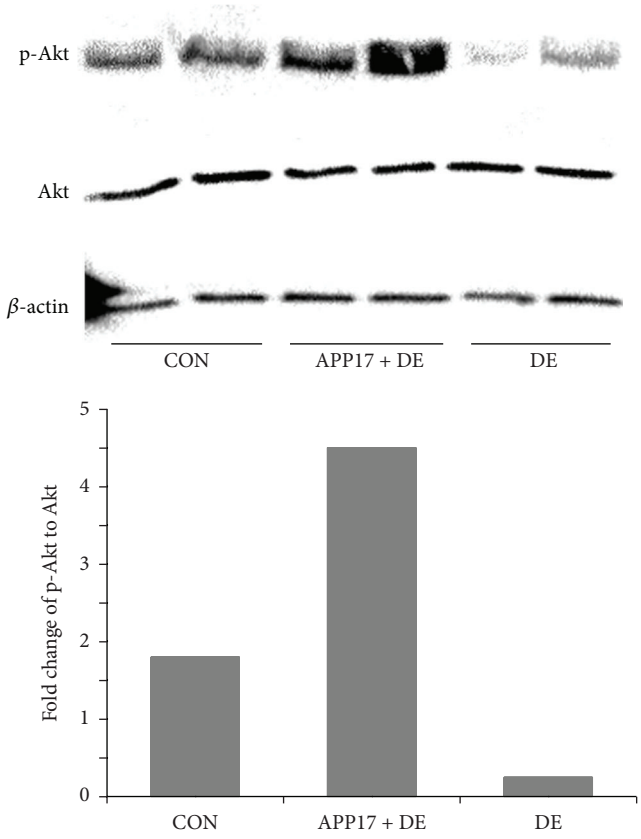

Figure 3: Enhanced insulin-induced Akt activation in the hippocampal gyrus of DE rats with APP17 treatment.

Compared with DE group, the expression of GLUT4 was obviously increased in the rat hippocampal gyrus in DE + APP17 group ($P < 0.05$) (Figure 4).

3.5. PI3K-Akt Pathway in Promoting Cell Glucose Metabolism by the APP17 Peptide. To identify the role of the PI3K-Akt pathway in the treatment of APP17 peptide, two specific inhibitors for the pathway were investigated in a glucose uptake assay *in vitro*. The Akt inhibitor 124011, a cell permeable and reversible benzimidazole compound, inhibits Akt phosphorylation/activation by targeting the ATP binding site of a kinase upstream of Akt but downstream of PI3K. Unlike phosphatidylinositol analog-based Akt inhibitors 124005, this inhibitor does not affect PI3K. PC-12 cells treated with the inhibitors or control were serum-starved for 24 h and then restimulated with insulin up to 20 h. The control cells and the PC-12 cells treated with the inhibitors were assayed for

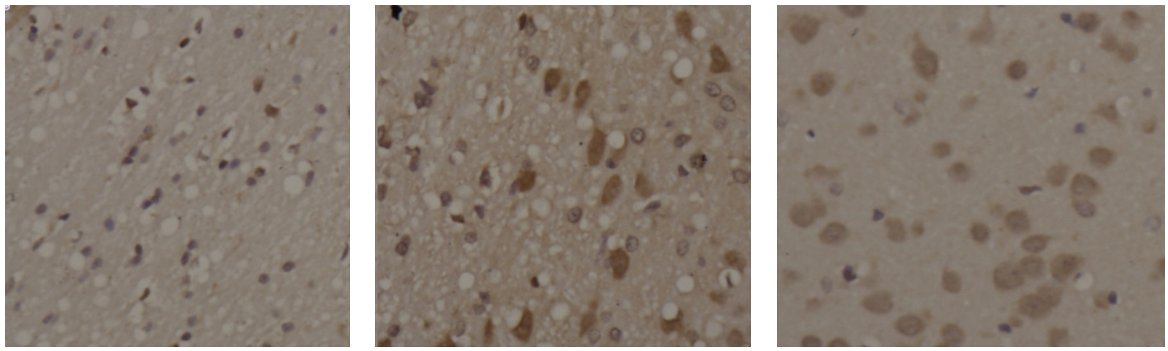

Figure 4: Enhanced GLUT4 expression in the hippocampal gyrus of DE rats with APP17 treatment.

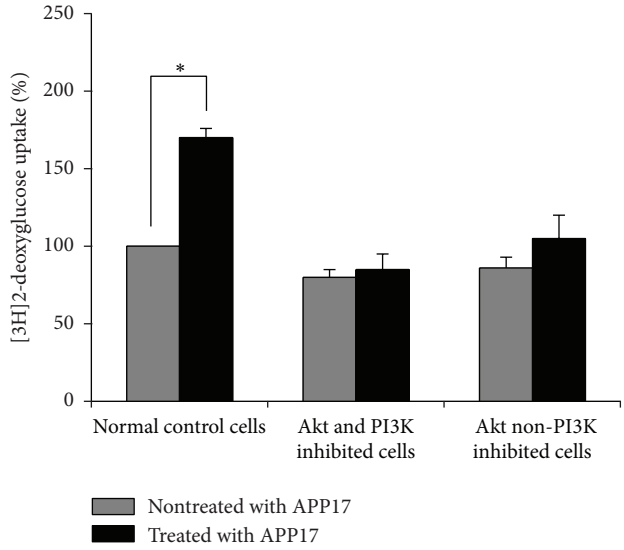

Figure 5: Enhanced insulin-induced glucose transport in PC-12 cells treated with APP17 peptide via PI3K-Akt pathway. Akt and PI3K inhibited cells pretreated with Akt inhibitor 124005; Akt non-PI3K inhibited cells were pretreated with Akt inhibitor 124011.

$[^3H]_2$-deoxy-D-glucose uptake after 16 h of insulin treatment. The group with the pretreatment of the inhibitors (Akt and PI3K inhibited cells and Akt non- PI3K inhibited cells) did not receive insulin stimulation. The incorporated radioactivity was determined by liquid scintillation counting of each sample in triplicate. Glucose uptake was not significantly altered in PC-12 cells treated with the inhibitors but was induced by 1.7-fold in normal control PC-12 cells (Figure 5).

All inhibitors that were used in the current study may affect both Akt1 and Akt2. According to Coleman's report, Akt2 plays an important role as a signaling molecule in the insulin signaling pathway. It is required to induce glucose transport. In a mouse model null for Akt1, but normal for Akt2, glucose homeostasis is unperturbed; however, the animals are smaller, consistent with a role for Akt1 in growth, and vice versa [22]. Hence it is inferred that APP17 peptide improves glucose metabolism in PC-12 cell via the Akt2-PI3K pathway.

No amyloid plaques in the cortex, nor the hippocampus, were detected in any of the groups. This indicates that the experimental animals in this study are not suffering from Alzheimer's disease, so the main reason affecting memory and learning ability is the diabetic encephalopathy.

At present, there are no effective drugs for the treatment of DE. This study showed that increased blood glucose levels were restored to normal in DE rats after treatment with APP17 peptide. This suggests that APP17 peptide acts by regulating glucose metabolism. The authors' laboratory has previously shown that APP17 peptide improves learning, memory function, and blood sugar concentration in STZ-induced DE rats and ameliorated the neurodegeneration of hippocampal neurons. This suggests that this peptide does not ameliorate DE through an unidentified action on a neuronal signal transduction pathway, rather through an insulinoid action. Previous reports have shown that cross-coupling between insulin and its receptors exists in human neuroblastoma cells [23].

These results show that glycometabolism plays an important role in the onset and development of neurodegenerative diseases and that the administration of APP17 peptide has neuroprotective effects against the changes induced by abnormal glycometabolism. APP17 peptide may cause these effects through the activation of common intracellular signaling pathways and initiation of "cross-talk" with neurotrophins. Further investigation is required to determine the mechanism by which APP17 peptide induces neuroprotection in this rat model. This may assist in identifying APP17 peptide as a potential therapeutic for neurodegenerative diseases.

4. Conclusions

The results of the current study indicate that APP17 peptide has a comprehensive therapeutic effect on diabetic encephalopathy, particularly through improving glycometabolism.

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Research Article

The Effect of Diabetes-Associated Autoantigens on Cell Processes in Human PBMCs and Their Relevance to Autoimmune Diabetes Development

Jana Vcelakova,[1] Radek Blatny,[2] Zbynek Halbhuber,[2] Michal Kolar,[3] Ales Neuwirth,[4] Lenka Petruzelkova,[1] Tereza Ulmannova,[1] Stanislava Kolouskova,[1] Zdenek Sumnik,[1] Pavlina Pithova,[5] Maria Krivjanska,[2] Dominik Filipp,[4] and Katerina Stechova[1]

[1] *Department of Paediatrics, 2nd Faculty of Medicine, Charles University in Prague and University Hospital Motol, V Uvalu 84, 15006 Prague, Czech Republic*
[2] *Central European Biosystems, Nad Safinou II 365, 25242 Vestec, Czech Republic*
[3] *Laboratory of Genomics and Bioinformatics, Institute of Molecular Genetics AS CR, Prague, Czech Republic*
[4] *Department of Immunobiology, Institute of Molecular Genetics, Czech Academy of Science, Videnska 1083, 14220 Prague, Czech Republic*
[5] *Department of Internal Medicine, 2nd Faculty of Medicine, Charles University in Prague and University Hospital Motol, V Uvalu 84, 15006 Prague, Czech Republic*

Correspondence should be addressed to Jana Vcelakova; jana.vcelakova@fnmotol.cz

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Type 1 Diabetes (T1D) is considered to be a T-helper- (Th-) 1 autoimmune disease; however, T1D pathogenesis likely involves many factors, and sufficient tools for autoreactive T cell detection for the study of this disease are currently lacking. In this study, using gene expression microarrays, we analysed the effect of diabetes-associated autoantigens on peripheral blood mononuclear cells (PBMCs) with the purpose of identifying (pre)diabetes-associated cell processes. Twelve patients with recent onset T1D, 18 first-degree relatives of the TD1 patients (DRL; 9/18 autoantibody positive), and 13 healthy controls (DV) were tested. PBMCs from these individuals were stimulated with a cocktail of diabetes-associated autoantigens (proinsulin, IA-2, and GAD65-derived peptides). After 72 hours, gene expression was evaluated by high-density gene microarray. The greatest number of functional differences was observed between relatives and controls (69 pathways), from which 15% of the pathways belonged to "immune response-related" processes. In the T1D versus controls comparison, more pathways (24%) were classified as "immune response-related." Important pathways that were identified using data from the T1D versus controls comparison were pathways involving antigen presentation by MHCII, the activation of Th17 and Th22 responses, and cytoskeleton rearrangement-related processes. Genes involved in Th17 and TGF-beta cascades may represent novel, promising (pre)diabetes biomarkers.

1. Introduction

Type 1 Diabetes (T1D) is considered to be a T-helper- (Th-) 1 autoimmune disease and is characterised by a lack of insulin, which is caused by the autoimmune destruction of insulin-producing pancreatic beta cells [1, 2]. Th1 lymphocytes are responsible for the infiltration of the islets of Langerhans and for the cytokine release that facilitates the destruction of beta cells by cytotoxic (Tc) lymphocytes. Due to this progressive damage, there is either insufficient or no production of insulin, leading to the first clinical signs of T1D. At the first appearance of clinical symptoms, most notably those associated with hyperglycaemia, nearly 80% of the beta cells have been destroyed, rendering the individual dependent on insulin injections [2, 3].

In patients presenting with recent T1D onset, there are various interventions that may stop, or at least delay, pancreatic beta cell destruction; however, these therapies

are unable to reverse the patient's lifelong dependency on insulin injections because beta cell proliferation and their capacity for regeneration are limited. To save sufficient beta cell masses, these therapies should be used in the clinically silent prediabetes phase; however, it is difficult to identify suitable candidates for such immunointervention [4–6].

The preclinical period is marked by the presence of autoantibodies against beta cell antigens, including insulin, glutamic acid decarboxylase-65 (GAD65), insulinoma-associated tyrosine phosphatase (IA-2), and zinc transporter 8 (ZnT8). The presence of these autoantibodies in the serum is highly predictive of T1D development [7–9]. However, the presence of autoantibodies alone is not sufficient to induce the destruction of beta cells [10–13].

The preclinical disease stage is characterised by the generation of activated, self-reactive lymphocytes that infiltrate the pancreas and selectively destroy the insulin-producing beta cells present in the islets [14]. In addition, other cellular immune mechanisms including immunoregulation and antigen presentation and processing are involved in T1D pathogenesis. Other studies have revealed the importance of the failure of regulatory mechanisms, which mainly include regulatory T cells, which suppress proliferation and cytokine production by both $CD4^+$ and $CD8^+$ T cells *in vitro* in a cell contact-dependent manner, and the secretion of anti-inflammatory cytokines (e.g., interleukin- (IL-) 10 and transforming growth factor- (TGF-) beta) [15]. Taken together, T1D pathogenesis is very complex, and all aspects of this disease are not fully understood. Although autoantibody detection is very helpful in the study of this disease, this method is not sufficient for the identification of a prediabetic person.

Autoreactive T lymphocytes are present in peripheral blood at extremely low frequencies, and methods for their detection are still used for scientific, rather than clinical, purposes [10, 13].

The last decade has ushered in a boom of "array techniques" that enable complex analyses of gene expression or protein production. These methods have also been used in T1D research to improve the prediction of T1D and increase the general knowledge of T1D pathogenesis [16, 17].

In this paper, we report the identification of cell processes that may be important for the progression of prediabetes to diabetes. We isolated peripheral blood mononuclear cells (PBMCs) and subsequently stimulated these cells with a mixture of "T1D-associated" autoantigens. We compared the expression profiles of stimulated PBMCs and PBMCs that were cultivated for the same period in the absence of autoantigens to determine the effect of autoantigens on gene expression. We describe, at the level of gene expression, the differences in the immune responses among the tested groups that are predicted to be important in T1D pathogenesis. Genes involved in these cascades, or in the activation of these cascades, may serve as promising potential prediabetes biomarkers. In our analyses, we primarily concentrated on functional pathways and attempted to reveal differences in gene expression among the multitude of signalling pathways within which these genes operate.

Table 1: Study population.

Study group	No. of individuals	Age (years) median, range	Age (years)	Sex (F/M)
T1D recent onset	12	12; 3–41	12	M
			12	M
			3	F
			17	M
			12	F
			8	F
			7	M
			41	M
			19	M
			15	F
			7	F
			7	F
First-degree relatives autoantibodies negative	9	19; 5–52	5	F
			7	F
			32	F
			52	M
			43	F
			19	F
			22	M
			16	M
			8	F
First-degree relatives autoantibodies positive	9	7; 3–21	7	F
			13	F
			10	M
			7	F
			3	M
			7	F
			7	F
			21	F
			7	F
Controls	13	27; 14–42	14	M
			36	F
			22	F
			22	F
			21	F
			21	M
			27	F
			31	M
			27	M
			42	M
			27	F
			32	F
			24	M

2. Materials and Methods

2.1. Study Subjects and Ethics. The study population is described in Table 1. Sera from all relatives were examined by radio-immune assay as a part of the national T1D prediction programme (RIA; Solupharm, Brno, Czech Republic) for the presence of autoantibodies against the islet antigens GAD65, IA-2, and insulin. The sample was considered positive if there was >1 IU/mL for GAD65 (GADA) and IA-2 (IA-2A) (>99th pct.). For insulin autoantibodies (IAAs), the cut-off was 0.4 U/mL. Autoantibody examination was successfully evaluated by the DASP 2010 (Diabetes Autoantibody Standardisation Programme of the Immunology of Diabetes Society). The

type of autoantibody positivity in sera from patients and relatives is indicated in Supplementary Table 1s available online at http://dx.doi.org/10.1155/2013/589451. Sera from healthy volunteers were autoantibody negative.

The sampling of patients with a recent T1D onset was performed after the metabolic stabilisation phase on the seventh day after diabetes diagnosis in the morning hours. Metabolic stabilisation is defined as the establishment of normoglycaemia and the normalisation of acid-base balance, biochemical parameters (such as ions and pH), and blood count parameters. Patients with severe diabetic ketoacidosis ($pH \leq 7.1$) at the time of the disease diagnosis were excluded from the study. The ethical approval, as well as the informed consent form, obligatory for all participants of this study, was processed by the Ethical Committee of the University Hospital Motol with respect to common national and EU rules. The patient's informed consent included blood sampling, isolation and analysis of nucleic acids, and anonymous data processing.

2.2. Cell Isolation and Stimulation by "T1D-Associated" Autoantigens. Approximately 17 mL of peripheral blood was obtained from the test subjects. PBMCs were isolated from whole venous blood by Ficoll density gradient centrifugation (Amersham Biosciences, Uppsala, Sweden) and were used in all *in vitro* experiments. The freshly isolated PMBCs (4×10^6 cells) were resuspended in 2 mL of RPMI-1640 Medium (Invitrogen, Carlsbad, CA, USA) supplemented with 20% foetal calf serum (FCS-F7524, Sigma-Aldrich, St. Louis, USA) and 10 μL/mL of Sigma solution, which contains 200 μM L-glutamine, 100 U penicillin, and 100 μg/mL streptomycin (G1146, Sigma-Aldrich, St. Louis, USA), and were cultured for 72 hours in the absence or presence of a mixture of the following autoantigen peptides (ProImmune, Oxford, UK): GAD65 amino acids (a.a.) 247–279 (NMYAMMIARFKM-FPEVKEKGMAALPRLIAFTSEE-OH), molecular weight 3,823.7; a.a. 509–528 (IPPSLRTLEDNEERMSRLSK-OH), molecular weight 2,371.7; a.a. 524–543 (SRLSKVAPVIKA-RMMEYGTT-OH), molecular weight 2,238.7; IA-2 a.a. 853–872 (SFYLK (Nleu) VQTQETRTLTQFHF), molecular weight 2,489; and a.a. 9–23 of β proinsulin (SHLVEALYLVCGERG), molecular weight 1,645 at a concentration of 2 μg/mL per $2 * 10^6$ PBMCs for all autoantigen peptides. Length and amount of antigen exposure were optimising in laboratory (data not shown).

2.3. Nucleic Acid Isolation and Gene Expression Microarrays. Total RNA from cultured cells was extracted using TRIzol reagent and a RiboPure kit (Invitrogen, Carlsbad, CA, USA), dissolved in 60 μL nuclease-free water and stored at −80°C. RNA concentration was measured using a spectrophotometer (Helios γ, Thermo Fisher Scientific, Waltham, MA, USA), and RNA integrity was assessed using an Agilent 2100 bioanalyser (Agilent, Palo Alto, CA, USA). To obtain a sufficient amount of RNA for the microarray assays, total RNA was amplified (aRNA) using the Amino Allyl MessageAmp II aRNA Amplification Kit (Applied Biosystems/Ambion, Foster City, CA, USA). The amplification procedure included the incorporation of 5-(3-aminoallyl)-UTP (aaUTP) into the aRNA during *in vitro* transcription to enable coupling of the RNA to N-hydroxysuccinimidyl ester-reactive Cy dyes. Twenty-five micrograms of aRNA was labelled with Cy3 or Cy5 dye. The Cy3 and Cy5 dyes were used to label RNA derived from nonstimulated and autoantigen-stimulated cells, respectively. From 3 to 6 μg of labelled aRNA was hybridised to a chip (two colour experimental settings), according to the protocol of the manufacturer. Samples were then processed using a high-density human whole genome HOA gene array (Phalanx Biotech, Palo Alto, CA, USA) that contains 32,050 probes with 30,968 human genome targets and 1,082 experimental control probes. The slides were scanned using InnoScan 700 (Innopsys, Carbonne, France) at 5 μm resolution. Artefacts were masked, and raw data were extracted using Mapix (Innopsys, Carbonne, France).

2.4. Gene Expression Microarray Data Analysis and Statistics. Microarray data processing and statistical analysis of differential gene expression was performed using the limma package in the R statistical environment (http://bioinf.wehi.edu.au/limma/), and a pathway analysis was performed with MetaCore (GeneGo, Inc., St. Joseph, MI, USA; http://www.genego.com/). Two-colour microarray data processing was performed as recommended by the array manufacturer. For each chip, raw intensity data were corrected for background, normalised by intra-array loess normalisation and subjected to subsequent interarray quantile normalisation. Differential gene expression was tested using the Bayesian moderated t-test in the limma package.

We examined differences in gene expression and affected cellular pathways between all combinations of the three groups: normal controls, diabetic patients, and their relatives, who were divided according to their autoantibody statuses. We compared basal gene expression with gene expression following stimulation with the diabetogenic autoantigens. The top table genes according to limma analysis (P value ≤ 0.05) were analysed by MetaCore to examine the functional relationships between the top genes (those genes with the most significant P values). We concentrated on identifying differences between tested pairs of study groups.

MetaCore is a proprietary, manually created database that analyses human protein-protein, protein-DNA, and protein-compound interactions, metabolic and signalling pathways, and the effects of bioactive molecules. This software generates interactive networks between user inputs and proteins and/or genes stored in the database. The software enables a user to analyse the distribution of canonical pathways, networks, GeneGo, and Gene Ontology processes, as well as the relevance of disease biomarkers in the tested samples. Canonical pathway maps represent a set of approximately 2,000 signalling and metabolic maps, comprehensively covering human biology. The content of approximately 110 cellular and molecular processes has been defined and annotated as GeneGo processes, and each process represents a preset network of interactions characteristic to the process. In this database, there are also over 500 human diseases with gene

content annotated by GeneGo and organised in disease-specific folders, which are further organised into a hierarchical tree (http://www.genego.com/). We were interested in the general enrichment analysis and in the involvement of selected genes in immune processes, for which the data were filtered in the MetaCore Biomarker Assessment Workflow.

2.5. qRT-PCR. qRT-PCR was used to verify microarray data. Differences in the expression levels of CD4, signal transducer and activator of transcription 3 (STAT3), and TGF-beta 1 between RNA samples from PMBCs collected from an independent cohort of T1D and the controls were assessed. Specifically, expression was analysed in a cohort of 14 newly diagnosed patients with T1D (7M/7F, mean age 8,6 years, median 9,1, range 1,7–17,2 years) and 12 control volunteers (5 M/7F, mean age 10,7 years, median 11,2, range 2,1–18,7 years) using TaqMan Gene Expression assays (Lifetechnologies, Carlsbad, CA, USA). Total RNA was extracted using TRIzol reagent, according to the manufacturer's recommendations (Lifetechnologies, Carlsbad, CA, USA). cDNA was synthesised according to recommendations by Lifetechnologies using the High Capacity RNA-to-cDNA Master Mix (Lifetechnologies, Carlsbad, CA, USA). Experiments were analysed using a LightCycler 480 Real-Time PCR System (Roche, Basel, Switzerland). A comparative ΔΔ cycle threshold (Ct) was used for quantification of relative mRNA levels. The expression of CD4 using commercially available primers (cat. no. Hs01058407_m1), STAT3 (cat. no. Hs00427259_m1), and TGF-beta (cat. no. Hs00171257_m1) was normalised to beta-glucuronidase (GUSB, cat. no. Hs99999908_m1).

Data from the qRT-PCR were analysed using the R programme. An unpaired, two-tailed Student's t-test was used for statistical analysis. Differences with a P value ≤ 0.05 were considered significant.

3. Results

3.1. Expression of Single Genes. Table 2 summarises the number of genes identified as having different expression levels when the various test groups were subjected to pair group comparisons. In the comparison of patients with T1D versus controls, statistically significant differences were present in the expression of 1,318 genes. The 20 genes that demonstrated the greatest changes in gene expression (up- or downregulated) are listed in Supplementary Table 2s. Interestingly, one of the most significantly upregulated genes in patients with T1D was CD4, a critical Lck-binding coreceptor required for the efficient activation of $CD4^+$ T cells [18]. Using qRT-PCR, the differential expression of CD4 was confirmed on a separate cohort of newly diagnosed patients with T1D and healthy controls (Figure 1). In addition, TGF-beta and STAT3, representatives of Th17 cell differentiation signalling (which scored as the second most significantly changed immune-related pathway in T1D patients compared to healthy controls), were also confirmed to be significantly ($P < 0.05$) upregulated (Figure 1).

Interestingly, the highest number of differentially expressed genes (2,222; P value ≤ 0.05) was found between relatives of TD1 patients and patients. A list of the top 20 up- and downregulated genes identified can be found in Supplementary Table 2s. Moreover, the relatives had significant alterations in the expression of 1,347 genes compared to controls (Supplementary Table 2s). However, we were unable to find any additional significant differences in gene expression when the relatives were divided according to autoantibody status in the DRLP (autoantibody/ies positive) and DRLN (autoantibody/ies negative) groups.

Table 2: The number of identified genes with different expression levels when the various test groups were subjected to pair group comparisons.

Comparison	Total no. of sign. differentially activated genes	No. of sign. upregulated genes	No. of sign. downregulated genes
DRL versus D	2222	1513	709
DV versus D	1318	896	422
DRL versus DV	1347	955	392

D: T1D patients; DRL: first-degree relatives of T1D patients; DV: controls (healthy volunteers).

An enhanced gene expression heatmap was constructed using probe signal intensities that had a log fold change that was greater than +1 or less than −1 (Figure 2).

3.2. Functional Genomics. The top ten canonical pathways that changed most significantly in the pair-wise comparisons are listed in Table 3(a) (summary), and Table 3(b) shows the complete list of significant immune response pathways identified for each pair-wise comparison.

The greatest number of differences for pathways that were altered, specifically 69 pathways, was observed when relatives were compared to controls. Of these pathways, 15% belonged to "Immune response pathways." However, the highest percentage (24%) of significant differences in immune response-related pathways was observed when patients with T1D were compared with healthy controls (11 out of 46 pathways), with "Antigen presentation by MHCII" as the highest scoring pathway. An important variable appeared to be Th17 lymphocyte activation, as we observed a difference in "Th17 cell differentiation" among the groups. Specifically, differences in Th17 polarisation were observed when relatives were compared with patients. The Th17 cell differentiation pathway is shown in Figure 3. Additionally, by comparing patients with T1D with the control group, we observed the distinct activation of important immune pathways involved in specific immune responses, such as Th1/Th2 polarisation, the formation of immunological synapses, and signalling via the T cell receptor (Table 3(b)).

Immunologic responsiveness in relatives was similar to the responsiveness observed in patients with T1D. However, only 7% of the differentially activated pathways could be classified as "immune response-related" (i.e., 4 pathways out of 54 differentially activated pathways). Within these pathways, cell cascades related to Th17 polarisation and the action of the immunoregulatory cytokine TGF-beta were also identified.

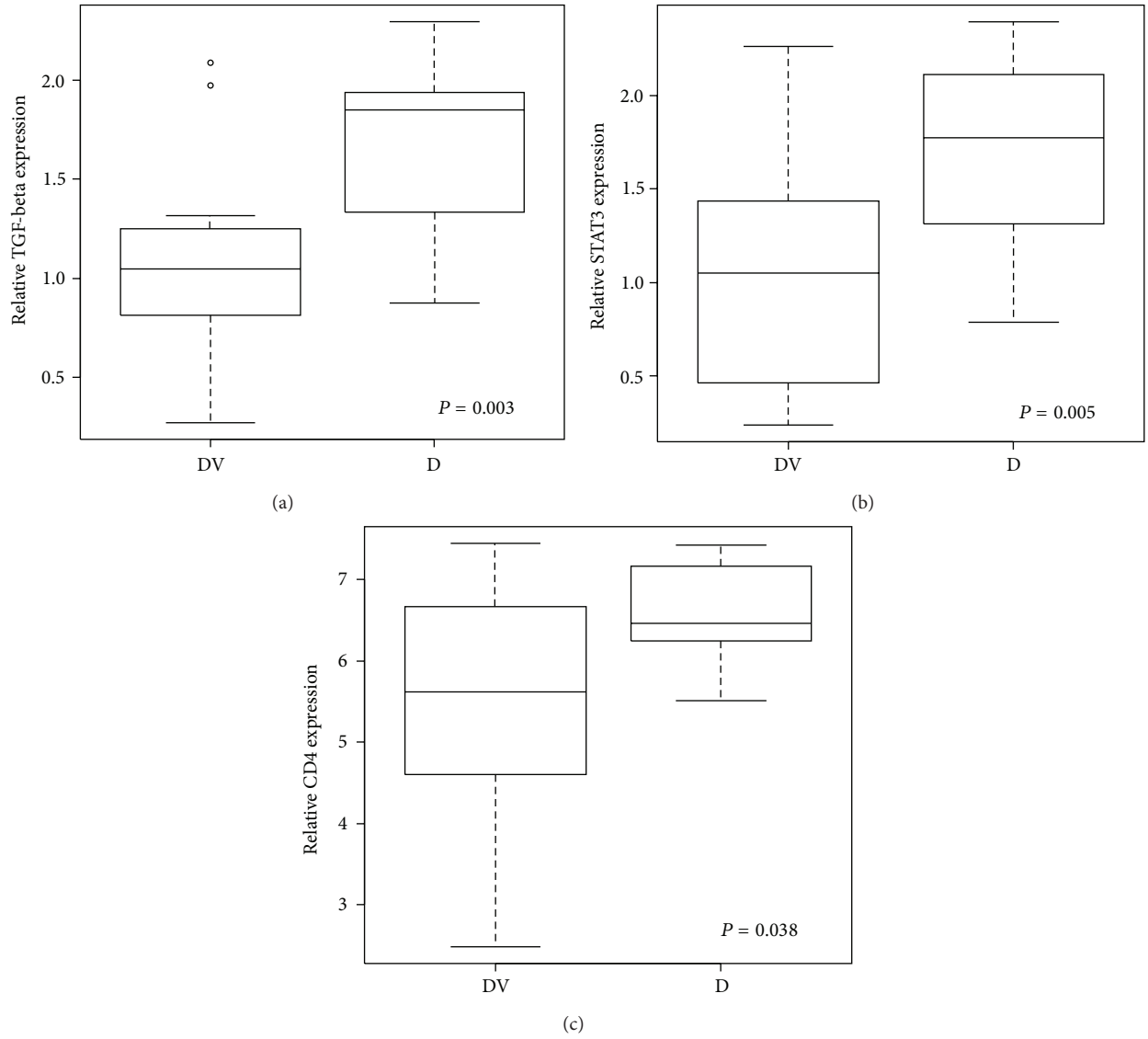

Figure 1: Verification of gene microarray data. Relative expression of TGF beta1, STAT3, and CD4 by qRT-PCR (data were obtained from an independent cohort of 14 newly diagnosed patients with T1D and 12 healthy volunteers).

4. Discussion

Upon activation and expansion, naive $CD4^+$ T cells develop into different Th cell subsets that exhibit different cytokine profiles and effector functions to protect the body against different types of pathogens. Until recently, T cells were divided into Th1 and Th2 cells, depending on the cytokines they produced (e.g., IFN-gamma and TNF-beta versus IL-4, -5, and -13, resp.).

A third subset of IL-17-producing effector Th cells called Th17 cells has recently been discovered. The participation of TGF-beta in Th17 cell differentiation places the Th17 lineage in close relationship with $CD4^+CD25^+Foxp3^+$ regulatory T cells (Tregs) [19].

T1D is an autoimmune disease that results from the selective destruction of pancreatic beta-cells by T cells, and the development of this disease is most likely due to the interaction between environmental and genetic factors. $CD4^+$ T cells are largely implicated in the pathogenesis of this disease, and T1D is believed to be a predominantly Th1-driven disease. Moreover, increased IL-17 expression has been detected in the sera and target tissues of patients with various autoimmune diseases, and in animal models, IL-23, a Th17 stabilisation factor, is involved in the development of autoimmune diabetes. The differentiation of Th17 cells is initiated by TGF-beta, IL-6, and IL-21, which activate STAT3 and induce the expression of transcription factors, including retinoic acid related orphan receptor (RORgamma t). In humans, Th17 activity seems to cause multiorgan inflammation, contributing to the manifestation of rheumatoid arthritis, inflammatory bowel disease, and celiac disease [20].

In this unique study on gene expression and functional analysis, we demonstrated that the "Th17 differentiation," "IL-22 signalling," and "Development of TGF-beta receptor

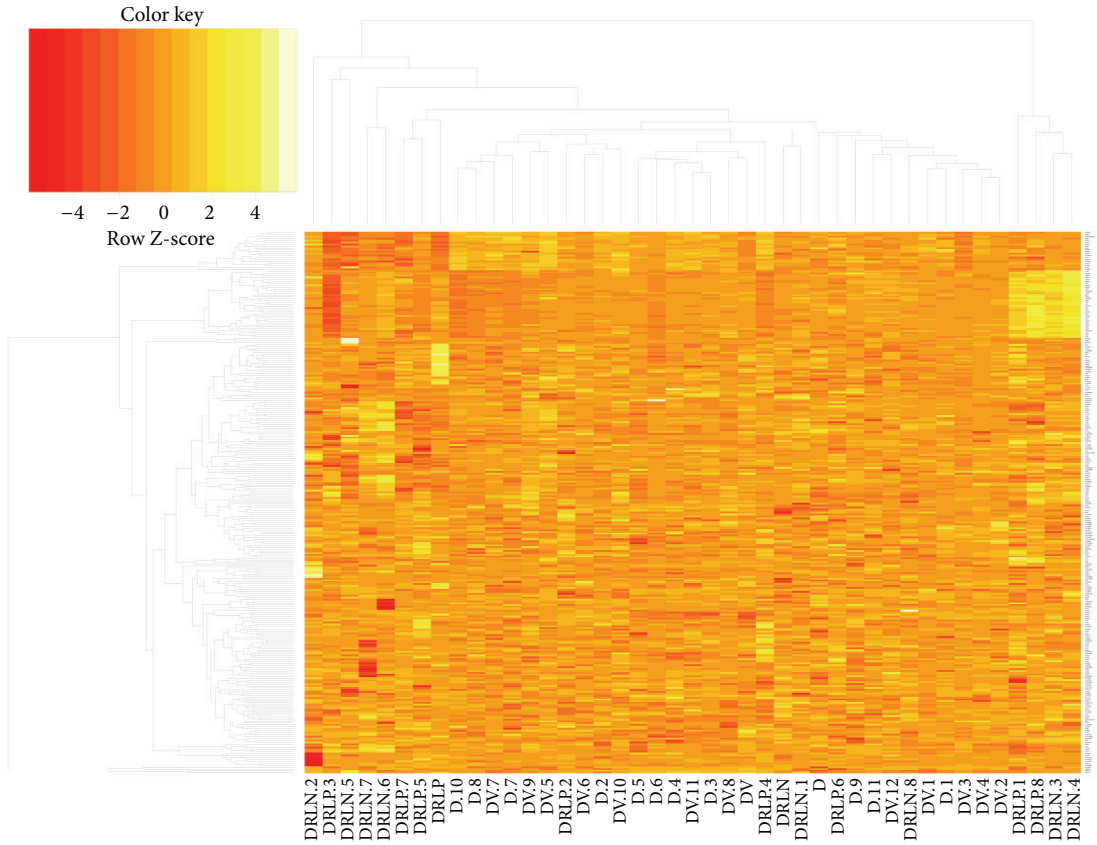

Figure 2: Genes differentially activated in each group (cluster formation). The enhanced gene expression heatmap was constructed using probe signal intensities that had a log fold change that was greater than +1 or less than −1. Genes that were significantly altered in the relatives group clustered into specific gene families.

signalling" pathways were among the most significantly different pathways identified when patients with T1D were compared with healthy controls. A difference in "Th17 signalling" pathway activation was also observed when we compared T1D patients with relatives. Consistent with these data, we previously reported that a bias in IL-10 and TGF-beta production at the protein level is typical of the prediabetes phase [21, 22].

Using a murine model of the disease, two groups previously reported that the transfer of islet-specific Th17 cells induced diabetes, although this effect was apparent only after the cells had converted to IFN-producing cells [23, 24]. Although TGF-beta and IL-21 can cause naive $CD4^+$ cells to differentiate into Th17 cells that secrete IL-17 in humans, it has been demonstrated that central memory $CD4^+$ cells can be driven to secrete IL-17 by a combination of IL-1 and IL-6 [25–28]. Bradshaw and colleagues studied monocytes directly isolated from the blood of patients with T1D and found that the cells spontaneously secreted the proinflammatory cytokines IL-1 beta and IL-6, which are known to induce and expand Th17 cells. Moreover, these *in vivo* activated monocytes induced more IL-17-secreting cells from memory T cells compared to monocytes from healthy control subjects. The induction of IL-17-secreting T cells by monocytes from patients with T1D was reduced *in vitro* with a combination of an IL-6-blocking Ab and an IL-1R antagonist. In this study, the authors also reported a significant increase in the frequency of IL-17-secreting cells in lymphocytes from long-term patients with T1D compared to healthy controls. These data suggest that the innate immune system in T1D patients may drive the adaptive immune system by expanding the Th17 population of effector T cells [29]. Consistent with the results of this report, our data also suggest that a "Th17 bias" may be present many years after disease onset and indicate the existence of a certain "autoreactive potential" of the immune system.

IL-9 is a T cell-derived cytokine that was initially characterised as a Th2 cytokine. The secretion of IL-9 was recently attributed to a novel $CD4^+$ T cell subset termed Th9 cells in mice. However, IL-9 can also be secreted by mouse Th17 cells and may mediate aspects of the proinflammatory activities of Th17 cells [30]. Beriou and colleagues reported that IL-9 is secreted by human naive $CD4^+$ T cells in response to differentiation under Th9- (i.e., TGF-beta and IL-4) or Th17- (i.e., TGF-beta and IL-6) polarising conditions. Yet, these differentiated naive cells did not coexpress IL-9 and IL-17 unless the cells were repeatedly stimulated under Th17 differentiation-inducing conditions. These authors demonstrated that patients with autoimmune diabetes exhibit higher frequencies of memory $CD4^+$ T cells and that activation of

Table 3: (a) GeneGo pathway top 10 maps ("immune response pathways" are in bold). (b) The complete list of significant "immune response pathways" for each pair comparison; pathway rankings (the position of each pathway within the list) are indicated.

(a)

T1D (D) patients versus healthy controls (DV)	Relatives of T1D patients (DRL) versus healthy controls (DV)	T1D (D) patients versus relatives of T1D patients (DRL)
(1) Immune response_Antigen presentation by MHCII	**(1) Immune response_MIF-JAB1 signalling**	(1) Cytoskeleton remodeling_CDC42 in cellular processes
(2) G protein signalling_Rac3 regulation pathway	(2) Cytoskeleton remodeling_Fibronectin bindings integrins in cell motility	(2) Development_BMP signalling
(3) Neurophysiological process_Olfactory transduction	(3) Translation_(L)-selenoaminoacids incorporation in proteins during translation	(3) Neurophysiological process_EphB receptors in dendritic spine morphogenesis and synaptogenesis
(4) Transcription_CREM signalling in testis	(4) Regulation of lipid metabolism_Insulin regulation of glycogen metabolism	(4) Development_Hedgehog signalling
(5) Dichloroethylene metabolism	(5) Glutathione metabolism	(5) Neolacto-series GSL Metabolism p.2 and p.3
(6) Delta508-CFTR traffic/sorting endosome formation in CF	(6) Development_Ligand-dependent activation of the ESR1/AP1 pathway	(6) Neurophysiological process_Olfactory transduction
(7) Immune response_Th17 cell differentiation	(7) G protein signalling_Rac3 regulation pathway	(7) Atherosclerosis_Role of ZNF202 in regulation of expression of genes involved in Atherosclerosis
(8) G-protein signalling_Regulation of CDC42 activity	(8) Protein folding_Membrane trafficking and signal transduction of G-alpha	(8) Dichloroethylene metabolism
(9) Immune response_IL-22 signalling pathway	(9) Neurophysiological process_Olfactory transduction	(9) Cytoskeleton remodeling_Neurofilaments
(10) Development_BMP signalling	(10) Dichloroethylene metabolism	(10) Triacylglycerol metabolism p.2

(b)

T1D (D) patients versus healthy controls (DV)	Relatives of T1D patients (DRL) versus healthy controls (DV)	T1D (D) patients versus relatives of T1D patients (DRL)
1: Antigen presentation by MHCII	1: MIF-JAB1 signalling	
7: Th17 cell differentiation	27: CXCR4 signalling via second messenger	
9: IL-22 signalling pathway	28: CXCR4 signalling pathway	
20: TCR and CD28 costimulation in activation of NF-kB	32: Regulation of T cell function by CTLA-4	24: Cytokine production by Th17 cells
23: Th1 and Th2 cell differentiation	36: IL-7 signalling in T lymphocytes	31: TGF-beta receptor signalling
25: HTR2A induced activation of cPLA2	43: IL-7 signalling in B lymphocytes	36: Th17 signalling pathway
28: IL-13 signalling via JAK-STAT	53: T cell receptor signalling pathway	40: Gastrin in inflammatory response
32: Lectin induced complement pathway	55: CD28 signalling	
33: Development of TGF-beta receptor signalling	56: Role of DAP12 receptor in NK cells	
35: T cell receptor signalling pathway	59: Immunological synapse formation	
41: Immunological synapse formation		

D: T1D patients; DRL: first-degree relatives of T1D patients; DV: controls (healthy volunteers).

these cells in the presence of TGF-beta induces a memory $CD4^+$ T cell response that is dominated by IL-9 and IL-17, accompanied by a loss of Th1 and Th2 cytokines. These data demonstrate that the presence of IL-9^+ IL-17^+ $CD4^+$ T cells induced by IL-1 beta may play a role in human autoimmune disease [30].

Not surprisingly, the highest scoring pathway in the comparison of patients with T1D versus their healthy counterparts was "Antigen presentation by MHCII"; indeed, it is well known that genes encoding HLA class II molecules are the most important "T1D-associated genes" [10]. It is also not surprising that other pathways related to crucial processes of the specific immune response, such as the "T cell receptor signalling pathway," demonstrated differences in activation in patients with T1D. Similarly, significant differences in Rho family GTPase signalling, namely, the Rac3 and Cdc42 pathways, which regulate cytoskeletal organisation and membrane trafficking and have been proposed to be linked to diabetes [31], were among the top ten pathways scored.

Glucose-stimulated insulin secretion from islet beta-cells involves secretory granule transport, a highly coordinated process that involves changes in cytoskeletal architecture with the help of G proteins and their respective effector molecules. Small G proteins include Cdc42, Rac1, and ARF-6, with corresponding regulatory factors including GDP/GTP-exchange factors and GDP-dissociation inhibitors. In addition to their positive modulatory roles, certain small G proteins also contribute to the metabolic dysfunction and the demise of islet beta-cells that has been observed in *in vitro* and *in vivo* models of impaired insulin secretion and diabetes [32].

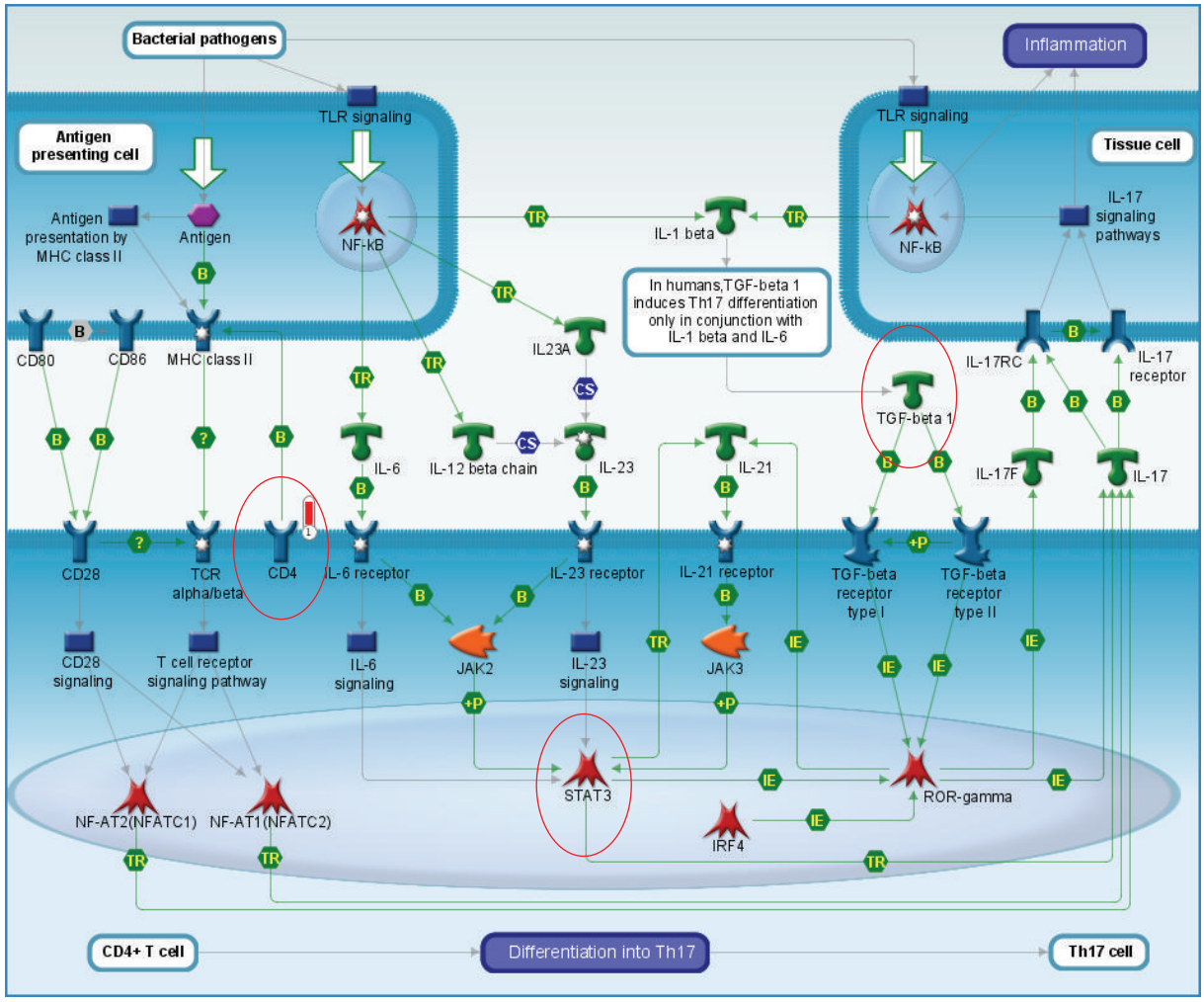

Figure 3: Immune response and Th17 cell differentiation. Differences in Th17 polarisation were observed when controls were compared with T1D patients using microarray data. Genes of interest were analysed by qRT-PCR and were found to be upregulated in T1D patients. STAT3 and TGF-beta were chosen as representatives of Th17 cell differentiation. Microarray data demonstrated that CD4 was one of the most significantly upregulated molecules in T1D patients.

The bone morphogenic protein (BMP) signalling pathway also appeared on the list of differentially activated pathways when patients were compared with controls and also with relatives. It is well known that diabetic nephropathy is a leading cause of end-stage renal disease. Additionally, the TGF-beta-BMP pathway has been implicated in the pathogenesis of diabetic nephropathy. The BMP2, BMP4, and BMP7 genes are located near linkage peaks for renal dysfunction, and it was hypothesised that genetic polymorphisms in these biological and positional candidate genes may constitute a risk factor for diabetic kidney disease; however, common BMP gene polymorphisms do not strongly influence genetic susceptibility to diabetic nephropathy in white individuals with T1D [33]. None of the tested patients had diabetic nephropathy at the time of sampling, but there may be a correlation between these symptoms and a higher risk of the development of chronic diabetic complications. Recently, it has also been suggested that TGF-beta/BMP-6 signalling in diabetic patients contributes to enhanced cell differentiation of circulating smooth muscle progenitor cells [34].

There have been only a limited number of T1D gene expression studies. One example is the report by Kaizer and colleagues [16] who analysed the gene expression of PBMCs derived from paediatric patients with T1D and T2D. The authors found that T1D and T2D likely share a downstream common pathway for beta-cell dysfunction that includes secretion of IL-1 beta and prostaglandins by immune effector cells, although the authors did not test the effect of autoantigen stimulation. In the Czech Republic, T2D is rare in children; therefore, we did not compare our data with data obtained from T2D patients, who typically belong to a more aged population. Reynier and colleagues tested first-degree relatives of T1D patients, but these authors also did not incorporate autoantigen exposure into their experiments, similar to Kaizer et al. [16]. Thus, our study appears quite unique in the sense that it compares the effects of autoantigen stimulation on cell processes in PMBCs in the normal and autoimmune diabetes states.

One potential drawback to our study is the limited number of samples tested. However, we believe that approximately

ten subjects per group are sufficient to reveal genes with statistically significant alterations in their gene expression levels when high-density microarray chips are used. In this context and in many other aspects, the results of this study parallel our previous work [35] and the studies of other research teams in which microarray analyses obtained from a limited number of subjects provided highly relevant and statistically significant data [16, 36–38]. Moreover, while our control group was not ideally age-matched to our other study groups, this variable produces negligible effects on our statistical analyses (data not shown) according to our comprehensive statistical analysis described elsewhere [35]. As an example, our assessment of the impact of age and sex on the expression of CD4 was statistically insignificant.

In conclusion, we can summarise that important differences were observed when the activation of cell processes following artificial exposure to diabetes-related autoantigens was compared among T1D patients, their first-degree relatives, and healthy controls. Important immune response-related pathways were involved, with a high degree of variability observed for these pathways when either patients with T1D or their relatives were compared with healthy controls. These important immune response-related processes largely included the induction of Th17 and Th22 responses, as well as cytoskeletal rearrangements, MHCII presentation, and the upregulation of CD4, TGF-beta, and STAT3. These findings potentially suggest that these processes could be utilised as predictive markers for the development of T1D or as molecular targets for the repression of specific immunocompetent cell populations for the treatment of diabetes.

Legend for the Tables and Figures

D:	T1D patients
DRL:	First-degree relatives of T1D patients
DRLN:	Relatives of T1D patients who are autoantibody(ies) negative
DRLP:	Relatives of T1D patients who are autoantibody(ies) positive
DV:	Controls (healthy volunteers)
FC:	Fold change
GADA:	Antiglutamic acid decarboxylase (GAD65) autoantibodies
IA-2A:	Antityrosin phosphatase (IA-2) autoantibodies
IAA:	Insulin autoantibodies.

Authors' Contribution

Radek Blatny, Zbynek Halbhuber, Michal Kolar, Dominik Filipp, and Katerina Stechova contributed equally to this work.

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Review Article

The Role of Uncoupling Proteins in Diabetes Mellitus

Jing Liu,[1] Ji Li,[2] Wen-Jian Li,[1] and Chun-Ming Wang[3]

[1] *Institute of Modern Physics, Chinese Academy of Sciences, Lanzhou 730000, China*
[2] *Department of Pharmacology and Toxicology, School of Medicine and Biomedical Sciences, University at Buffalo, State University of New York, Buffalo, NY 14214, USA*
[3] *School of Life Sciences, Lanzhou University, Lanzhou 730000, China*

Correspondence should be addressed to Chun-Ming Wang; cmwang@lzu.edu.cn

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Uncoupling proteins (UCPs) are anion carriers expressed in the mitochondrial inner membrane that uncouple oxygen consumption by the respiratory chain from ATP synthesis. The physiological functions of UCPs have long been debated since the new UCPs (UCP2 to 5) were discovered, and the role of UCPs in the pathogeneses of diabetes mellitus is one of the hottest topics. UCPs are thought to be activated by superoxide and then decrease mitochondrial free radicals generation; this may provide a protective effect on diabetes mellitus that is under the oxidative stress conditions. UCP1 is considered to be a candidate gene for diabetes because of its role in thermogenesis and energy expenditure. UCP2 is expressed in several tissues and acts in the negative regulation of insulin secretion by β-cells and in fatty acid metabolism. UCP3 plays a role in fatty acid metabolism and energy homeostasis and modulates insulin sensitivity. Several gene polymorphisms of UCP1, UCP2, and UCP3 were reported to be associated with diabetes. The progress in the role of UCP1, UCP2, and UCP3 on diabetes mellitus is summarized in this review.

1. Introduction

Diabetes mellitus (DM) is a chronic metabolic disease which is characterized by hyperglycemia, absolute or relative deficiencies in insulin secretion, and/or insulin action. The new classification proposed by the American Diabetes Association in 1997 was based on the pathogenesis of the disease and comprises four categories: Type 1 DM (DM1), Type 2 DM (DM2), other types, and gestational diabetes [1]. DM1 is mostly due to genetic disorders and an autoimmune disease resulting in the selective destruction of β-cells in the pancreas that leads to insulin loss. DM2, also called noninsulin-dependent diabetes mellitus (NIDDM), is characterized by insulin resistance with significant metabolic dysfunction including obesity, impaired insulin function and secretion, and increased endogenous glucose output. Although the two types of diabetes have distinct etiologies, they lead to similar diabetic complications, and both of them are related to oxidative stress status. It is demonstrated that activation of oxidative stress pathways plays a key role in the development of not only the late complications (such as cardiovascular disease, nephropathy, retinopathy, and amputations) in DM1 and DM2, but also in the early stage such as insulin resistance [2]. There are many sources of reactive oxygen species (ROS) production in diabetes including mitochondrial and nonmitochondrial origins: NADPH oxidase, xanthine oxidase, uncoupled eNOS, lipoxygenase, cyclooxygenase, cytochrome P450 enzymes, and other hemoproteins [3], and mitochondrion is thought to be the main source of ROS generation site in DM. The mechanisms about ROS and DM development were shown in Figure 1.

The uncoupling proteins (UCPs) are a family of mitochondrial transport proteins located in the inner mitochondrial membrane. There are five UCPs (named UCP1 to 5) found in mammals [4]. These anion-carrier proteins transport protons (H^+) to the mitochondrial matrix and in turn dissipate the proton motive force as heat and uncouple the substrate oxidation from the production of ATP. These proteins have similarities in their structures, but different tissue distributions in mammals. UCP1 is mainly expressed in brown adipose tissue (BAT), which is responsible for

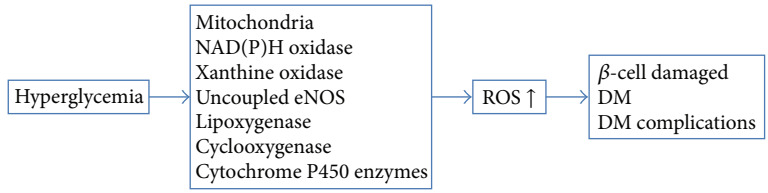

Figure 1: The mechanisms of ROS and DM development. Hyperglycemia may activate ROS generation through the mitochondrial and nonmitochondrial origins: NADPH oxidase, xanthine oxidase, uncoupled eNOS, lipoxygenase, cyclooxygenase, cytochrome P450 enzymes, and other hemoproteins. ROS can further cause β-cell damages and the development of DM and related complications.

thermogenesis in newborns. UCP2 is widely distributed in several tissues including the spleen, kidney, immune system, pancreas, and central nervous system, whereas UCP3 is mainly restricted to the skeletal muscle, and UCP4 and UCP5/BMCP1 are mainly expressed in the brain. Besides the nonshivering thermogenesis function of UCP1, functions of the other UCPs are still unclear. UCP2 is reported to be involved in glucose and lipid metabolism [5] to control immune cell activation by modulating MAPK pathways and the production of mitochondrial ROS [6], and a neuroprotective role is also suggested based on the regulation of mitochondria membrane potential, production of ROS, preservation of calcium homeostasis, modulation of neuronal activity, and eventually inhibition of cellular damage [7]. UCP3 is suggested to be involved in mediating energy expenditure via uncoupling, especially in fatty acid metabolism, and it seems to protect mitochondria against lipid-induced oxidative stress, which makes this protein a potential player in the development of DM2 [8]. Fewer studies focused on the physiologic roles of UCP4 and UCP5, and the protection against oxidative stress and mitochondrial dysfunction are also reported [9]. Although the physiological functions of UCPs are still not been completely elucidated, their abilities of reducing mitochondrial ROS formation are widely accepted [10]. That is, high membrane potential of mitochondria will induce ROS production and thus oxidative damage; these ROS may activate UCPs and therefore cause a "mild uncoupling" and (as a negative feedback) will prevent further superoxide production and decrease oxidative damage (Figure 2). This "antioxidative activity" of UCPs makes it logical to search any benefit on DM through counteracting the oxidative stress appeared in DM and the complications. On the other hand, it is also found that UCP2 is a negative regulator of insulin secretion; the superoxide-mediated activation of UCP2 causes pancreatic β-cell dysfunction [11].

The vast majority of diabetes are Type 1 or Type 2 (DM2 represents at least 80 percent and DM1 accounts for about 5–10 percent), and increasing numbers of studies focused on the roles of UCPs on DM or the complications. We will review the progress in the relations between UCPs (UCP1, UCP2, and UCP3) and DM1 and/or DM2 in the following.

2. Role of UCP1 in DM

The relationship between UCP1 and DM has already been reported long before the other UCPs were discovered in 1997. Studies revealed that UCP1 mRNA and protein concentrations in BAT were regulated by insulin [12, 13]. As UCP1 has been proved to decrease membrane potential, downregulate ROS generation, and increase energy expenditure, so *UCP1* gene is regarded as a candidate gene for obesity, DM2, or related traits. The role of UCP1 in the development of obesity and DM2 has been reviewed in 2010 and 2012 [14, 15]; the authors focused on the polymorphisms −3826A/G, −1766A/G, and −112A/C in the promoter region, Ala64Thr in exon 2, and Met299Leu in exon 5 of *UCP1* gene and pointed out that they are possibly associated with obesity, lipid/lipoprotein-related disease, and/or DM2. The −3826A/G polymorphism of UCP1 was further reported to be associated with diabetic retinopathy (DR) in DM1 group and the *UCP1* gene expression was increased in human retina [16]; while the same polymorphism of UCP1 (−3826A/G) was not found the association with DM2 with European ancestry in another study [17], although a significant association between the UCP2 Ala55Val and UCP3 −55C/T polymorphisms and increased susceptibility for DM2 were detected in Asians in the same study [17]. The −3826A/G polymorphism influenced *UCP1* gene expression: G allele carriers had higher *UCP1* cDNA and protein concentrations than A/A carriers. And more interestingly, G allele carriers exhibited increased *MnSOD2* expression, which suggested that this allele could be a marker of oxidative stress. As oxidative stress is related to DR, so this deleterious polymorphism in *UCP1* gene is suggested to be a risk factor for DR (multivariate analysis confirmed that the G/G genotype was an independent risk factor for DR) [16].

UCP1 had been thought to be expressed only in rodents and human infants for a long time; however, UCP1 protein and/or its mRNA expression were detected in human white adipose tissue, skeletal muscle, longitudinal smooth muscle layers, retinal cells, and islet cells recently [18, 19], although the physiological functions of UCP1 in these tissues and organs are not established as well as in BAT. In 2013, the adult human neck brown fat is further reported with the anatomical localization, gene expression profiling, and functional characterization [20]. The imbalance between energy intake and expenditure is the underlying cause of obesity and DM. BAT consumes fuel for thermogenesis through tissue-specific UCP1; it was once thought that BAT had a functional role in rodents and human infants only, but it has been recently shown that in response to mild cold exposure adult human BAT consumes more glucose per gram than any other tissues

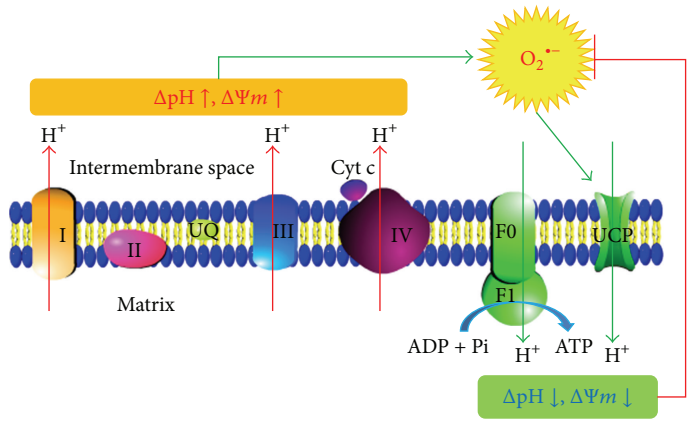

FIGURE 2: "Antioxidative activity" of UCP. High transmembrane proton gradient and membrane potential of mitochondria will induce ROS production and thus oxidative damage; these ROSs may activate UCPs and therefore cause a "mild uncoupling" and (as a negative feedback) will prevent further superoxide production and decrease oxidative damage. ΔpH: transmembrane proton gradient; $\Delta\Psi m$: mitochondrial membrane potential.

[21]. In addition to this nonshivering thermogenesis, human BAT may also combat weight gain by becoming more active in the setting of increased whole-body energy intake. This suggests that activation of human BAT could be used as a safe treatment for obesity and metabolic dysregulation and further help to cure DM. In view of the *UCP1* gene expressed in the other tissues, more attention may be paid to the role of UCP1 in muscle tissue, islet cells, and thymus function in the future.

3. Role of UCP2 in DM

UCP2 is the most widely distributed UCP and highly expressed in pancreatic β-cells in DM, so it is the most frequently studied one concerning its role in DM. Mitochondrial dysfunction and β-cell failure exhibited a close correlation in DM2. In β-cells, ROSs activate UCP2, which results in proton leak across the mitochondrial inner membrane, and this leads to reduced β-cell ATP synthesis and content, which is a critical parameter in regulating glucose-stimulated insulin secretion (GSIS) [22]. The recent reviews about UCP2 and DM were published in 2009 and 2011 [23–25]. We will review the progress acquired since then in the following.

3.1. UCP2 Is a Negative Regulator of Insulin Secretion. Early in 2001, just 4 years after the discovery of UCP2, it was reported that UCP2 negatively regulated insulin secretion and was a major link between obesity, β-cell dysfunction, and DM2 [26]. In a model of obesity-induced diabetes mice (ob/ob mice), UCP2 was markedly upregulated in islets. The UCP2-deficient mice had higher islet ATP levels and increased GSIS; this indicated that UCP2 negatively regulates insulin secretion. The ob/ob mice lacking UCP2 had restored first-phase insulin secretion, increased serum insulin levels, and greatly decreased levels of glycemia [26]. In recent years, the mitochondrial ROS generation or oxidative stress in β-cell was found to be a prominent role in the effect of UCP2 on insulin secretion, although the consequences were not always consistent. In a study published in 2009, all of the three highly congenic strain backgrounds (C57BL/6J, A/J, 129/SvImJ) Ucp2−/− mice exhibited increased oxidative stress, and the GSIS in Ucp2−/− islets of each congenic strain was significantly decreased [27]. UCP2 knockdown in INS-1E insulinoma cells improved the GSIS, and this can be annulled completely by the cell-permeative antioxidant MnTMPyP [28]. In the islets of β-cell-specific UCP2 knockout mice, the intracellular ROS levels were found elevated and GSIS enhanced [29]. All of these three studies described a ROS-related pathway about the role of UCP2 and GSIS. That is to say, the UCP2 knockdown will cause elevation of ROS and/or oxidative stress and then the enhancement of GSIS.

3.2. UCP2 Gene Polymorphisms Are Associated with DM. A study about the associations between polymorphisms in UCP2 and UCP3 with DM2 was carried out in Korea and found that the UCP2 −5331G>A and UCP3 −2078C>T polymorphisms are susceptibility markers for DM2 among Koreans [30]. Among the other three studies, no significant association of the UCP2 −866G/A polymorphism with DM2 risk was observed [31–33]. The study about Asian Indians indicated that Ala55Val polymorphism at UCP2 and −55C/T polymorphism at UCP3 are associated with a significantly reduced risk of developing DM2 [32]. While these correlations are different between Europeans and Asian descent: neither the UCP2 Ala55Val nor the UCP3 −55C/T polymorphism showed any significant association with DM2 risk in Europeans (OR 1.04, 95% CI 0.98, 1.09 for Ala55Val; OR 1.04, 95% CI 1.00, 1.09 for −55C/T); and in contrast, a statistically significant association was observed for both polymorphisms in participants of Asian descent (OR 1.23, 95% CI 1.12, 1.36 for Ala55Val; OR 1.15, 95% CI 1.03, 1.28 for −55C/T) [33].

3.3. UCP2 Gene Polymorphisms Are Associated with Other DM-Related Chronic Complications. In a study of the relationship between UCP2 polymorphisms and proliferative

diabetic retinopathy (PDR), three UCP2 polymorphisms were selected (−866G/A (rs659366), Ala55Val (rs660339), and 45 bp insertion/deletion (Ins/Del)), and the haplotype [A Val Ins] was an independent risk factor for PDR in both types 1 and 2 diabetic groups [34]. Three studies focused on the relationship between −866G/A polymorphism and obesity in a Balinese population, ischemic stroke in Chinese DM2 patients, and obesity in Danes and showed a significant association between them [35–37].

3.4. Many Compounds or Medicines Exhibit Curative Effect on DM through Downregulated UCP2 Gene Expression. American *Ginseng* stimulates insulin production and prevents apoptosis through downregulation of UCP2 in cultured β-cells [38]. Inhibited UCP2 expression by an antisense oligonucleotide can reverse diet-induced DM mice models by the effects on both insulin secretion and action [39]. MicroRNA-15a positively regulates insulin synthesis by directly targeting and inhibiting UCP2 gene expression [40]. A Chinese medicine, Kaiyuqingre formula, improves insulin secretion via decreasing the overexpression of UCP2 in cultured INS-1 cells [41]. Korean red *Ginseng* promoted the expression of insulin and downregulated the expression of UCP2 in the spontaneously diabetic Goto-Kakizaki rats [42].

Most of the studies about the role of UCP2 in DM focused on the UCP2 functions in β-cells, and the results indicated a deleterious effect of UCP2 on DM. As UCP2 is widely expressed in many tissues, the antioxidative activities (downregulating mitochondrial ROS generation) of this protein should be evaluated in the future.

4. Role of UCP3 in DM

Much fewer studies focused on the role of UCP3 in DM, for it was thought to be expressed restrictedly in skeletal muscle for a long time. UCP3 mRNA and protein levels are decreased in skeletal muscle of patients with DM2 compared to healthy control subjects. UCP3 protein content is reduced in prediabetic subjects (i.e., impaired glucose tolerance) and DM2, and eight weeks of rosiglitazone treatment significantly increases insulin sensitivity and restores skeletal muscle UCP3 protein in diabetic patients [43]. Similar to UCP2, UCP3 was found to be expressed in pancreatic β-cells in 2008, where it also influenced insulin secretion [44], although the physiological function of UCP3 in β-cells is still not known. UCP3 mRNA is expressed in human islets; the relative abundance of UCP2 mRNA was 8.1-fold higher ($P < 0.05$); immunohistochemical analysis confirmed colocalization of UCP3 protein with mitochondria in human β-cells. UCP2 protein expression in human islets was increased approximately 2-fold after high glucose exposure, whereas UCP3 protein expression was decreased by approximately 40% ($P < 0.05$). UCP3 overexpression improved glucose-stimulated insulin secretion. These results indicated that UCP2 and UCP3 may have distinct roles in regulating β-cell function; increased expression of UCP2 and decreased expression of UCP3 in humans with chronic hyperglycemia may contribute to impaired glucose-stimulated insulin secretion [44].

4.1. UCP3 Gene Polymorphisms Are Associated with DM. Early in the year 2000, a study carried out French Caucasians found the association between the −55C/T polymorphism of the *UCP3* gene and DM2; the study found that subjects bearing the TT genotype have a lower risk for developing DM2 than the others do [45]. This result is the same as reported in the Asian Indians cohort [32] but differs from another study in Europeans which could not find any significant association with DM2 risk [33]. These inconsistent results may be due to the different gene background between different human species. Another report about Finnish cohort indicated that the *UCP3* gene variant rs3781907 was associated with a higher risk of DM2 [46].

4.2. Relationship between UCP2-UCP3 Gene Cluster Variation and DM. The *UCP2* and *UCP3* genes are located within 8 kb of each other in a gene cluster on chromosome 11q13. Several genetic variants in the *UCP2-UCP3* gene cluster have been examined in multiple studies, including −866G/A (rs659366), Ala55Val (rs660339), a 45-bp insertion/deletion (I/D) in the 3′ untranslated region (UTR) of exon 8 in UCP2, and the −55C/T (rs1800849) polymorphism in UCP3, and the associations with obesity and/or diabetes were also reported. Many of these *UCP2* and *UCP3* gene polymorphisms (such as −866G/A, Ala55Val of UCP2, and −55C/T of UCP3) are also evaluated within *UCP2-UCP3* gene cluster variation studies; the difference between the polymorphisms and the cluster study may be just that the cluster variation studies are often focusing on many variations at the same time. A research published in 2006 evaluated the impact of the UCP2 −866G>A and UCP3 −55C>T variants on prospective risk of DM2 and found that these variations in the *UCP2-UCP3* gene cluster are associated with an increased risk of DM2 [47]. In 2008, a report focused on 14 tag single nucleotide polymorphisms (tSNP) (rs637028, rs653263, rs622064, rs2306820, rs673494, rs655717, rs643064, rs660339, rs659366, rs591758, rs668514, rs647126, rs1800006, and rs1800849), for each of the 14 tSNPs across the genomic region of the *UCP2-UCP3* gene cluster; they did not observe significant effects on DM2. However, Haplotype-based analyses suggest that a haplotype set defined by rs591758, rs668514, rs647126, and rs1800006 was significantly associated with DM2 risk in Caucasian women only, especially among those who were overweight [48]. This may be emphasizing that the DM2 development is driven by a multifactor model more than a single-factor one. In a Finnish diabetes prevention study, three variants in the *UCP2* gene, one variant in the *UCP2-UCP3* intergenic region, and five variants in the *UCP3* gene were explored [46]. The authors concluded that the genetic variations in the *UCP2-UCP3* gene cluster may act as a modifier increasing serum lipid levels and indices of abdominal obesity and may thereby also contribute to the metabolic aberrations observed in obesity and DM2. Another study analyzed other 14 tSNPs (rs622064, rs2306820, rs655717, rs660339, rs17132534, rs659366, rs668514, rs3741135, rs1626521, rs2734827, rs3781907, rs1800849, rs1685333, and rs826071) in *UCP2-UCP3* gene cluster and could not find an association of any of the 14 tSNPs tested with DM2 risk [49]. This result is similar to the other 14 tSNPs study mentioned

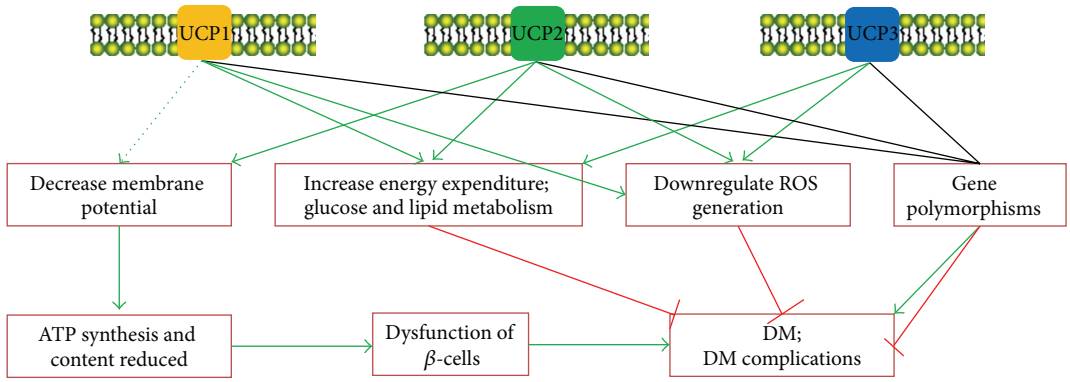

Figure 3: The role of UCPs on the development of DM and/or complications. UCPs may affect the development of DM through 4 aspects: decrease mitochondrial membrane potential, increase energy expenditure especially through glucose and lipid metabolisms, downregulate ROS generation, and gene polymorphisms. The green arrows represent pathways activation, the dotted green arrow means that this pathway needs to be further proved, and the red inhibition arrows represent the inhibition effect.

previously, and they may both imply the same fact that the development of DM2 is a multifactor style; any one of the 14 tSNPs is not enough to affect the DM2. It is important to illustrate how the polymorphisms of *UCP* genes are involved in the development of DM, and this may help to develop new strategies for DM prevention and/or treatment.

5. Conclusion and Future Directions

The present data indicated that all of the three UCPs (UCP1, UCP2, and UCP3) have some kind of relationships with the development of DM (Figure 3). As DM is a multifactorial disease, the ethnic differences, gender, genomic factors, age, nutritional characteristics, lifestyle, and even environmental factors are all related to the outcomes. The single UCP or single *UCP* gene variation may not be enough to affect the results. More importantly, the physiological function of UCPs, at least UCP2 and UCP3, is still unclear, which may limit this kind of study very much. So, it is better to keep on exploring the physiological function of UCPs in the future, and based on this, the synergetic studies on the multisite of UCPs variations may help to elucidate the relationship between UCPs and DM.

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Research Article

Alteration of 11β-Hydroxysteroid Dehydrogenase Type 1 and Glucocorticoid Receptor by Ethanol in Rat Liver and Mouse Hepatoma Cells

Zhaojie Meng,[1] Xueying Bao,[2] Ming Zhang,[1] Shengnan Wei,[1] Wenguang Chang,[1] Jing Li,[1] Li Chen,[1] and B. L. Grégoire Nyomba[3]

[1] *Department of Pharmacology, School of Norman Bethune Medical Sciences, Jilin University, Changchun, Jilin 130021, China*
[2] *The 208th Hospital of the Chinese People's Liberation Amry, Changchun, Jilin 130062, China*
[3] *Department of Internal Medicine, University of Manitoba, Winnipeg, MB, Canada R3E3P4*

Correspondence should be addressed to Jing Li; chensubmit@163.com

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Alcohol is a potential risk factor of type 2 diabetes, but its underlying mechanism is unclear. To explore this issue, Wistar rats and mouse hepatoma cells (Hepa 1–6) were exposed to ethanol, 8 g·kg^{-1}·d^{-1} for 3 months and 100 mM for 48 h, respectively. Glucose and insulin tolerance tests *in vivo* were performed, and protein levels of 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) and glucocorticoid receptor (GR) in liver and Hepa 1–6 cells were measured. Alterations of key enzymes of gluconeogenesis phosphoenolpyruvate carboxykinase (PEPCK) and glucose 6 phosphatase (G6Pase), as well as glycogen synthase kinase 3a (GSK3α), were also examined. The results revealed that glucose levels were increased, and insulin sensitivity was impaired accompanied with liver injury in rats exposed to ethanol compared with controls. The 11β-HSD1, GR, PEPCK, G6Pase, and GSK3α proteins were increased in the liver of rats treated with ethanol compared with controls. Ethanol-exposed Hepa 1–6 cells also showed higher expression of 11β-HSD1, GR, PEPCK, G6Pase, and GSK3α proteins than control cells. After treatment of Hepa 1–6 cells exposed to ethanol with the GR inhibitor RU486, the expression of 11β-HSD1 and GR was significantly decreased. At the same time the increases in PEPCK, G6Pase, and GSK3α levels induced by ethanol in Hepa 1–6 cells were also attenuated by RU486. The results indicate that ethanol causes glucose intolerance by increasing hepatic expression of 11β-HSD1 and GR, which leads to increased expression of gluconeogenic and glycogenolytic enzymes.

1. Introduction

During several decades, many cohort studies from the medical epidemiology literature have observed a close association between ethanol consumption and type 2 diabetes [1, 2]. Some studies have suggested that heavy drinking induces the development of type 2 diabetes and is a potential risk factor for diabetes; however, consuming moderate amounts of alcohol has been reported to reduce the incidence of diabetes [3]. So far, the relationship between alcohol and diabetes has not been well characterized. Notably, the mechanism of alcohol-induced diabetes remains uncertain.

Type 2 diabetes is a metabolic syndrome characterized by insulin resistance and decreased in insulin secretion [4, 5]. Full-blown type 2 diabetes is preceded by impaired glucose tolerance (IGT) and impaired fasting glucose (IFG) globally termed prediabetes, which is associated with an increased risk for the development of type 2 diabetes [6, 7]. Subjects with IFG have increased hepatic glucose output and early dysfunction of insulin secretion, while subjects with IGT have moderate-to-severe insulin resistance in the muscle [8, 9]. It is well known that phosphoenolpyruvate carboxykinase (PEPCK) and glucose 6 phosphatase (G6Pase) are the rate-limiting enzymes in hepatic gluconeogenesis, whereas glycogen synthase kinase 3 (GSK3) plays an important role in glucose production and storage [10–13].

As antagonists of insulin action, glucocorticoids are major sources of increased glucose production in type 2

diabetes though upregulation of key enzymes in gluconeogenesis. Excess tissue glucocorticoid action may contribute to the hyperglycemia and insulin resistance associated with type 2 diabetes. Inactive glucocorticoids (cortisone, 11-dehydrocorticosterone) are converted into active forms (cortisol, corticosterone) by 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) [14]. Active glucocorticoids bind to glucocorticoid receptor (GR), stimulate the expression of PEPCK and G6Pase, and enhance glucose production from both gluconeogenesis and glycogen degradation in liver [15].

It is well established that long-term excessive ethanol consumption impairs glucose tolerance, induces insulin resistance, and leads to the development of type 2 diabetes. Ethanol causes oxidative and endoplasmic reticulum stress in pancreatic β cells [16, 17], and this can result in impairment of insulin secretion [18]. In the present study, we investigated whether glucose tolerance is altered in association with 11β-HSD1 and GR in rats chronically treated with high amounts of ethanol corresponding to human chronic alcoholism.

2. Materials and Methods

2.1. Materials. Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin, and 0.25% trypsin EDTA solution were purchased from Gibco BRL (Grand Island, NY, USA). RU486 was purchased from Sigma (St. Louis, MO, USA). Insulin was purchased from Eli Lilly, Changchun, China. Glucose oxidase kit was obtained from Beijing BHKT Clinical Reagent Co., Beijing, China. [^{125}I]Insulin radioimmunoassay kit was purchased from Tianjin Nine Tripods Medical & Bioengineering Co., Tianjing, China. Polyclonal antibodies to 11β-HSD1, GR, PEPCK, G6Pase, GSK3α and actin, and goat anti-rabbit IgG horseradish peroxidase conjugate were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). GAPDH was purchased from Epitomics (Burlingame, CA, USA). ECL Western Blotting Substrate was purchased from Pierce (Thermo Fisher Scientific, Rockford, USA). Chemical reagents for western blot were obtained from Sigma and polyvinylidene difluoride membranes were from Bio-Rad (Hercules, USA).

2.2. Animal Experiments. Male Wistar rats (200–220 g) obtained from the Experimental Animal Holding Facility of Jilin University were randomly divided into two groups: normal control group and ethanol-treated group. After one week of acclimatization, the ethanol group was given 36% ethanol (8 g·kg^{-1}·d^{-1}) via an intragastric tube, and the control group was given an equal volume of water. This administration was carried out twice daily at 9 AM and 4 PM for three months. Both groups of rats were given free access to a normal chow and water. Body weight and food intake were recorded weekly. The protocols for animal care and handling were approved by the Animal Care and Use Committee of Jilin University.

2.3. Cell Culture. Mouse hepatoma (Hepa 1–6) cells obtained from ATCC were grown in DMEM supplemented with 10% fetal bovine serum, 1% L-glutamine (200 mM), penicillin (40 units·mL^{-1}), and streptomycin (40 μg·mL^{-1}). Hepa 1–6 cells were passaged using a 0.25% trypsin-EDTA solution, seeded at 1×10^6 cells·dish^{-1} in 3 mL DMEM with 10% FBS, and incubated at 37°C for 48 h. Dishes of cells were then randomly divided into 4 groups: (1) control, (2) control + RU486, (3) ethanol, and (4) ethanol + RU486. The cells in ethanol and ethanol + RU486 groups were treated with 100 mM ethanol refreshed every 12 h for 48 h. 10 μM of RU486, an inhibitor of GR, was added to the cells at the 24th h of ethanol incubation in control + RU486 and ethanol + RU486 groups for 24 h. RU486 was dissolved in ethanol to a stock concentration of 10 mM, which was diluted 1000 times with the culture media, and the same concentration of the solvent was used for control and ethanol groups.

2.4. Intraperitoneal Glucose Tolerance Test (IPGTT). IPGTT was conducted at 3 months of age after a 16 h fast using an i.p. glucose injection (2 g·kg^{-1}). Blood was taken by tail snip at 0, 30, 60, and 120 min after the glucose injection. Glucose concentration in serum samples was determined using a glucose oxidase kit.

2.5. Insulin Tolerance Test (ITT). ITT was performed at 3 months of age after a 12 h fast using an i.p. insulin injection (0.75 U·kg^{-1}). Blood was obtained by the same method as for IPGTT to measure glucose concentration.

2.6. Plasma Insulin. The rats were fasted for 16 h and blood was taken from the abdominal aorta under anesthesia with i.p. injection of urethane (1 g/kg body weight). Insulin concentration in each sample was measured by RIA, and plasma glucose concentration was determined using a glucose oxidase kit.

2.7. Western Blotting. Liver tissue and Hepa 1–6 cells were homogenized at 4°C in 1 mL or 500 μL of ice cold TES buffer (20 mM Tris-HCl, pH 7.4, containing 250 mM sucrose, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.01 mM leupeptin, and 5 μg·mL aprotinin) for 60 min, and the lysate was centrifuged at 10,000 rpm for 5 min at 4°C. Aliquots of the supernatant were removed for protein analysis by the Bradford method (Bio-Rad). The samples (160 μg proteins) were denatured by boiling for 5 min and separated by 10% SDS polyacrylamide gel electrophoresis and then electroblotted onto a polyvinylidene difluoride membranes (Bio-Rad) at 4°C. After blocking in 5% (w/v) nonfat milk for 2 h at room temperature, the membranes were incubated with respective rabbit polyclonal specific primary antibodies with gentle agitation overnight at 4°C. The membranes were washed 3 times for 10 min each with 15 mL of TBST (10 mM Tris-HCl, 150 mM NaCl, and 0.1% (v/v) Tween-20) and then incubated with a secondary antibody (1 : 2000 goat anti-rabbit IgG horseradish peroxidase conjugate) at room temperature for 2 h. The bands of proteins were visualized with ECL on a X-ray film. The protein bands were scanned and quantified using the Quantity One image analysis software (Bio-Rad).

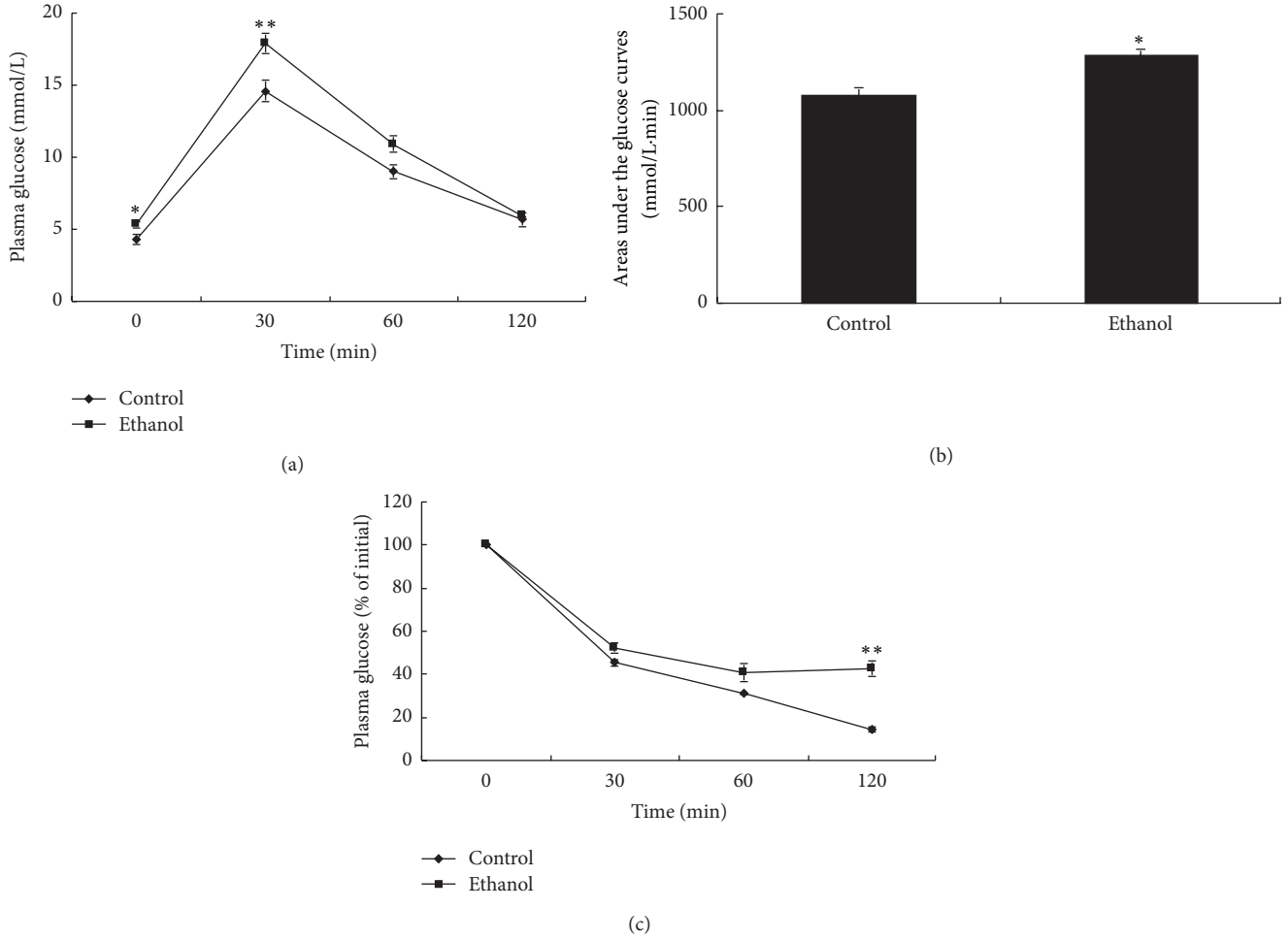

FIGURE 1: Glucose and insulin tolerance after 3 months of ethanol intake (8 g·kg^{-1}·d^{-1}). Plasma glucose concentrations during intraperitoneal glucose tolerance test (IPGTT), the area under the IPGTT glucose curve, and the glucose concentrations during insulin tolerance test (ITT) are shown as the mean ± S.E.M ($n = 20$). $^{*}P < 0.05$, $^{**}P < 0.01$ ethanol versus control.

2.8. Statistical Analysis. All data were expressed as the mean ± SEM. Statistical analyses were performed using t-test for significance using SPSS software (version 13.0 for Windows). $P < 0.05$ was considered to be significant.

3. Results

3.1. Effects of Ethanol on Body Weight and Metabolic Parameters in Rats. Mean body weight and food intake during the study period are summarized in Table 1. Body weight was not significantly different between control and ethanol groups. Average food intake of the 12 weeks in ethanol group (67.5 ± 0.53 g·kg^{-1}·d^{-1} [403.3 ± 3.17 cal·kg^{-1}·d^{-1}]) was slightly lower compared with controls (74.3 ± 1.03 g·kg^{-1}·d^{-1} [443.9 ± 6.15 cal·kg^{-1}·d^{-1}]). However, the amount of ethanol ingested (8 g·kg^{-1}·d^{-1}) provided 47.8 cal·kg^{-1}·d^{-1}, increasing the total caloric intake in Ethanol group to 451.1 cal·kg^{-1}·d^{-1}, which is similar to that of control group. Metabolic changes induced by ethanol are presented in Table 2. Ethanol-treated group had higher fasting blood glucose, total cholesterol, triglyceride, alanine aminotransferase, and aspartate aminotransferase levels compared with the control group ($P < 0.05$–0.01). Plasma insulin levels were reduced in ethanol-treated group ($P < 0.05$).

IPGTT and ITT were carried out in ethanol and control groups to more accurately determine glucose tolerance and insulin sensitivity (Figure 1). As shown by the IPGTT glucose curve, rats of the ethanol group had higher blood glucose compared with the control group, and the areas under the glucose curves (mmol·L^{-1}·min) were significantly greater in the ethanol-treated group compared with controls ($P < 0.05$). During insulin tolerance test (ITT), the glucose concentration declined slowly in ethanol-treated group, and at 120 min the glucose level (percentage of initial) was clearly higher in the ethanol group than in the control group ($P < 0.01$). This result demonstrated that 3 months of ethanol intake (8 g·kg^{-1}·d^{-1}) caused insulin resistance. Overall these data indicate that long-term ethanol intake can result in

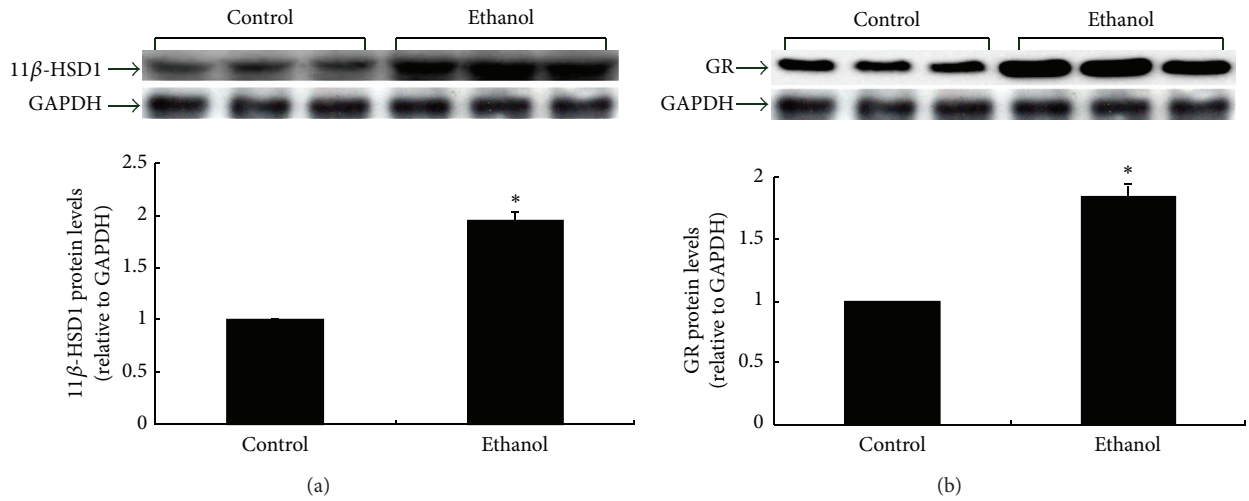

Figure 2: 11β-HSD1 and GR proteins in the liver of control and ethanol rats after 3 months of ethanol intake (8 g·kg^{-1}·d^{-1}). Protein levels are expressed relative to the control and shown as the mean ± SEM ($n = 6$). $^{*}P < 0.05$ ethanol versus control.

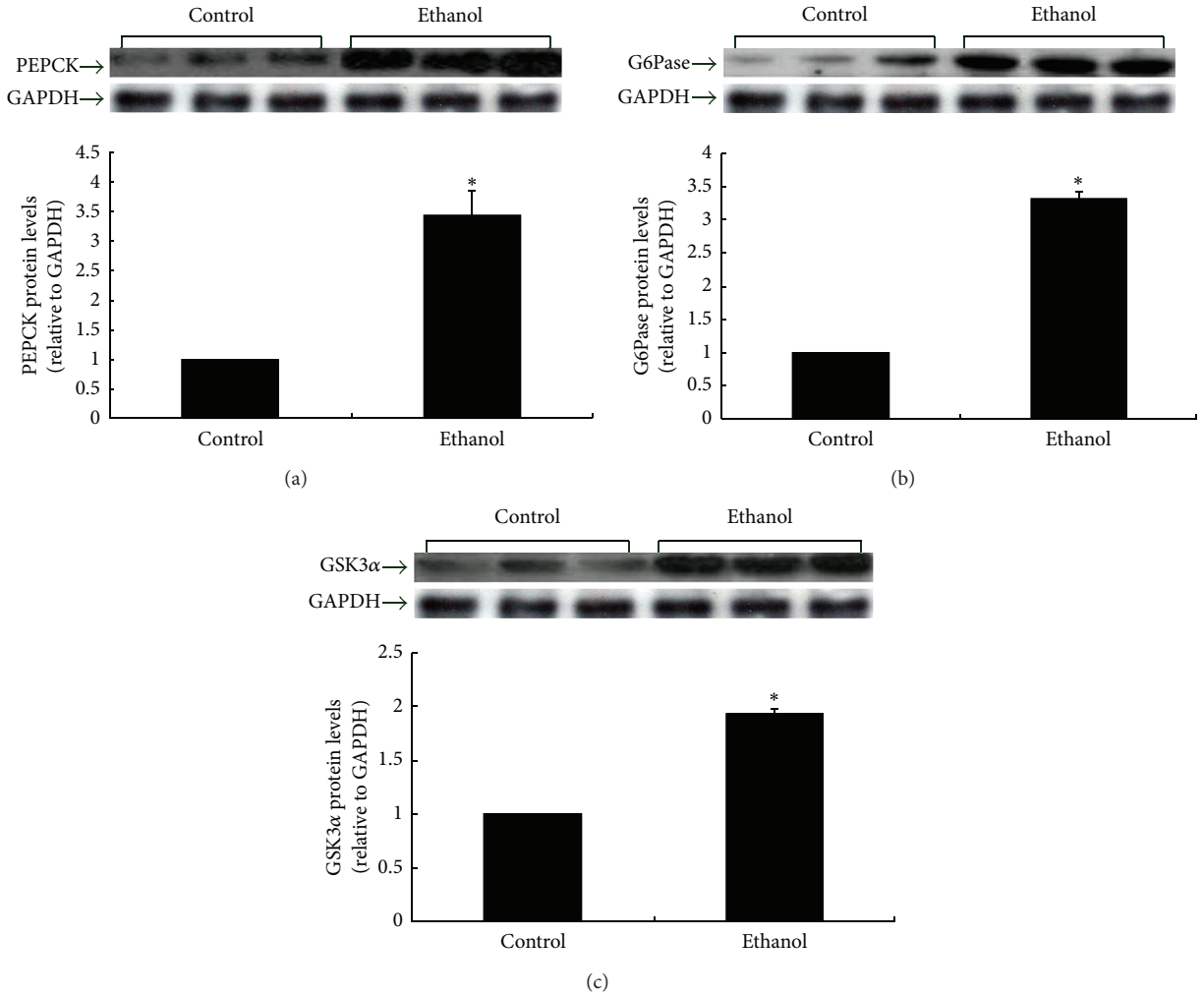

Figure 3: PEPCK, G6Pase, and GSK3α proteins in the liver of control and ethanol rats after 3 months of ethanol intake (8 g·kg^{-1}·d^{-1}). Protein levels are expressed relative to the control and shown as the mean ± SEM ($n = 6$). $^{*}P < 0.05$ ethanol versus control.

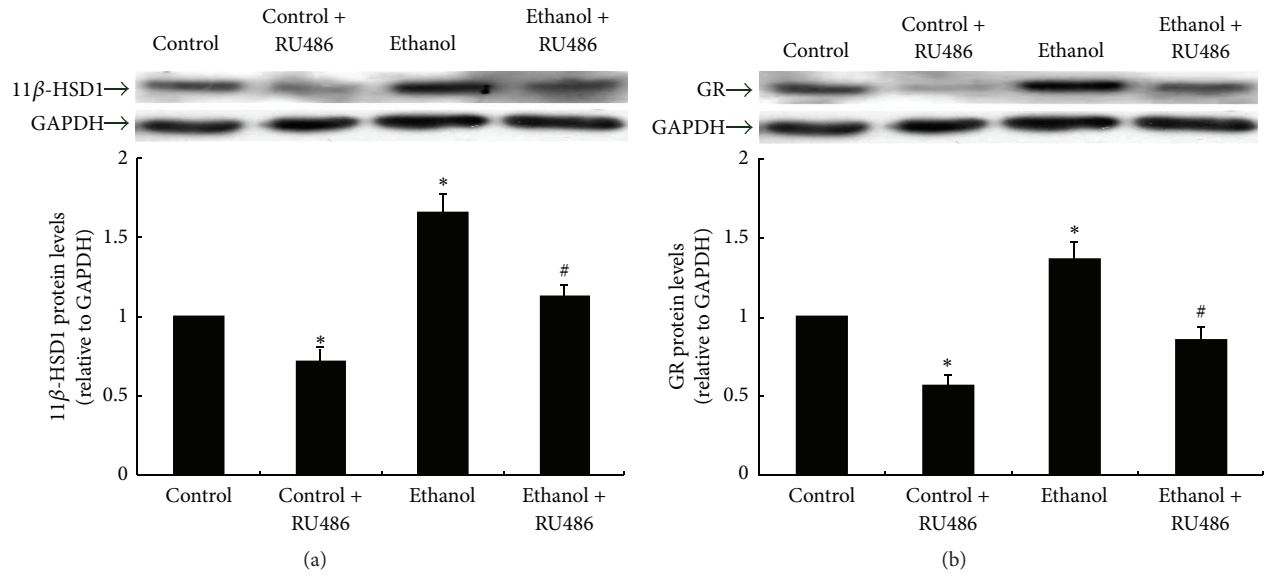

Figure 4: 11β-HSD1 and GR proteins in Hepa 1–6 cells. Groups of cells were treated with 100 mM ethanol and/or 10 μM RU486, as described in Section 2. Protein levels are expressed relative to control and shown as the mean ± SEM. ($n = 6$). $^{*}P < 0.05$ versus control; $^{\#}P < 0.05$ versus ethanol.

Table 1: Body weight and food intake of ethanol and control rats (mean ± SEM, $n = 20$).

Week	Body weight (g)		Food intake (g·kg^{-1}·d^{-1})	
	Control	Ethanol	Control	Ethanol
1	213 ± 5.4	214 ± 5.9	73.2 ± 2.34	66.4 ± 2.80
2	242 ± 5.8	239 ± 6.2	71.0 ± 2.89	68.2 ± 1.26
3	269 ± 7.1	254 ± 7.7	69.5 ± 1.85	68.5 ± 2.36
4	288 ± 4.6	277 ± 7.0	71.2 ± 2.43	69.7 ± 1.08
5	305 ± 7.6	299 ± 5.8	75.1 ± 2.62	67.2 ± 2.00
6	328 ± 6.0	319 ± 5.9	70.7 ± 2.43	69.9 ± 2.51
7	358 ± 9.1	346 ± 7.7	74.0 ± 1.11	67.6 ± 1.16
8	361 ± 8.9	358 ± 6.0	78.7 ± 2.49	69.3 ± 2.23
9	370 ± 8.8	371 ± 7.4	80.5 ± 0.81	67.7 ± 0.80
10	383 ± 9.4	380 ± 7.5	78.1 ± 1.56	63.9 ± 1.84
11	392 ± 9.6	381 ± 7.4	76.8 ± 0.76	66.4 ± 2.36
12	408 ± 9.6	391 ± 8.0	72.6 ± 1.22	65.1 ± 0.77

Table 2: Metabolic parameters of ethanol and control rats.

Group	Control	Ethanol
Fasting blood glucose (mmol·L^{-1})	4.31 ± 0.32	5.12 ± 0.25**
Fasting plasma insulin (mIU·L^{-1})	18.7 ± 2.56	12.1 ± 1.13*
Total cholesterol (mmol·L^{-1})	2.27 ± 0.07	2.57 ± 0.06*
Triglycerides (mmol·L^{-1})	1.54 ± 0.09	1.84 ± 0.07*
Alanine aminotransferase (U·L^{-1})	6.39 ± 0.04	9.98 ± 0.06**
Aspartate aminotransferase (U·L^{-1})	4.36 ± 0.03	7.54 ± 0.06**

Data are expressed as the mean ± S.E.M ($n = 20$).
$^{*}P < 0.05$, $^{**}P < 0.01$ ethanol versus control.

insulin resistance, glucose intolerance, and alteration of lipid metabolism.

3.2. Effects of Ethanol on 11β-HSD1 and GR Proteins in the Rat Liver. To investigate the alterations of 11β-HSD1 and GR in liver of rats after ethanol exposure, their protein levels were determined using western immunoblot (Figure 2). The protein level of 11β-HSD1 was significantly elevated in the liver of ethanol-treated rats compared with controls (Figure 2, $P < 0.05$). At the same time, the protein expression of GR was higher in the ethanol than in the control group (Figure 2, $P < 0.05$).

3.3. Effects of Ethanol on Major Gluconeogenic Enzymes and Glycogen Synthase Kinase 3 in Rat Liver. The expression of PEPCK and G6Pase, two rate-limiting enzymes in gluconeogenesis, was significantly increased in ethanol-treated rats compared with controls (Figure 3, $P < 0.05$), explaining at least in part the hyperglycemia of ethanol-treated rats. As well, the level of GSK3α was higher in ethanol-treated rats than in controls (Figure 3, $P < 0.05$). GSK3 inactivates glycogen synthase, which is the rate-limiting enzyme in glycogen synthesis, and overexpression of GSK3 decreases glycogen synthesis in liver and impairs glucose utilization.

3.4. Effects of Ethanol on 11β-HSD1 and GR in Hepa 1–6 Cells. Liver is one of the major organs responsible for glucose metabolism; therefore, we further examine if the observations in adult rats occur also in the hepatic cells (Hepa 1–6). Hepa 1–6 cells were treated with 100 mM ethanol refreshed every 12 h for a total of 48 h. After 24 h of ethanol treatment, 10 μM RU486 was added. Preliminary results using MTT assay demonstrated that Hepa 1–6 cell viability was not altered by this concentration of RU486 (data not shown). The protein level of 11β-HSD1 was significantly elevated in Hepa 1–6 cells treated with ethanol compared with control cells (Figure 4). The GR inhibitor RU486 remarkably reduced the

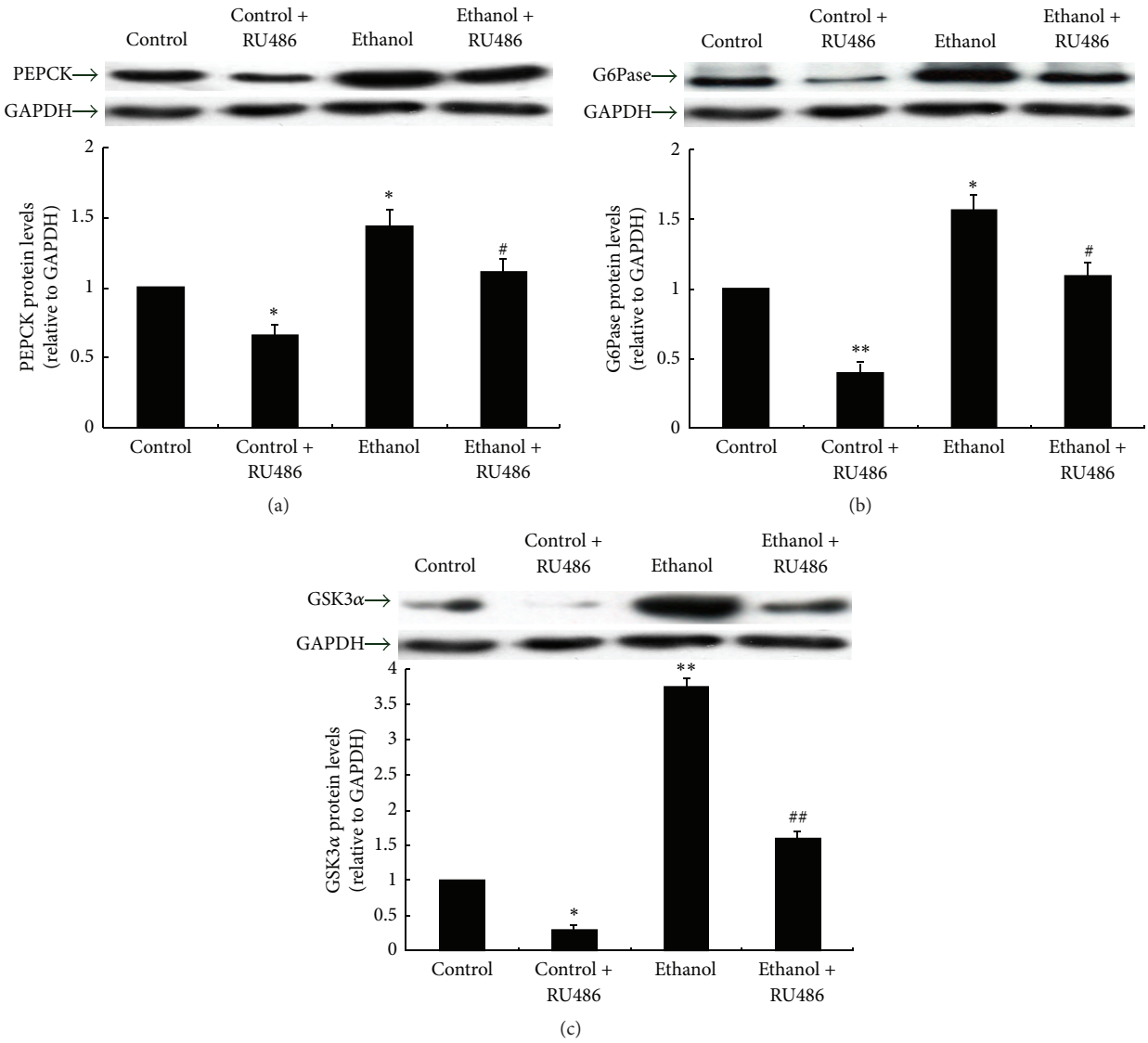

Figure 5: PEPCK, G6Pase, and GSK3α proteins in Hepa 1–6 cells. Groups of cells were treated with 100 mM ethanol and/or 10 μM RU486. Protein levels are expressed relative to the control and shown as the mean ± SEM. ($n = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$ versus control; $^{\#}P < 0.05$ versus ethanol.

protein expression of 11β-HSD1 in both control and ethanol-treated cells. The GR protein levels showed similar alterations (Figure 4).

3.5. Effects of Ethanol on Gluconeogenic Enzymes in Hepa 1–6 Cells. As observed in rat liver *in vivo*, the PEPCK protein level was significantly higher in ethanol-treated than control Hepa 1–6 cells ($P < 0.05$). In these cells, RU486 decreased the PEPCK protein expression (Figure 5). The protein expression of G6Pase presented similar changes (Figure 5).

3.6. Effect of Ethanol on Glycogen Synthase Kinase in Hepa 1–6 Cells. As shown in Figure 5, GSK3α protein level was markedly increased in Hepa 1–6 cells treated with ethanol, and RU486 reduced the GSK3α protein expression in Hepa 1–6 cells with or without prior ethanol treatment.

4. Discussions

Heavy ethanol consumption is a potential risk factor for type 2 diabetes. Human drinking alcohol at doses of 50–60 g·kg^{-1} twice per day develops type 2 diabetes [19, 20]. In the present study, rats given ethanol at 8 g·kg^{-1}·d^{-1} for 3 months had glucose intolerance and reduced insulin sensitivity in association with altered lipid regulation. The rats also had reduced fasting insulin levels, consistent with the suggestion that excessive ethanol causes pancreatic β cell dysfunction and apoptosis through oxidative and endoplasmic reticulum stress [16, 17]. The associations of elevated fasting glucose and insulin resistance suggest that ethanol causes alterations of glucose regulation leading to both IFG and IGT. Given these characteristics, the focus of the present research was on the effect of ethanol on enzymes regulating hepatic glucose

metabolism, as these could both explain insulin resistance and elevated fasting glucose. First, the rate-limiting enzymes in hepatic gluconeogenesis and glycogen synthesis involved in the development of type 2 diabetes were determined in rats exposed to ethanol. The results showed that alcohol consumption increased expression of PEPCK and G6Pase, which are key enzymes of gluconeogenesis. In addition, ethanol enhanced the protein expressions of hepatic GSK3α, one isoform of glycogen synthase kinase 3 (GSK3). GSK3 is a constitutively active kinase in resting cells that becomes rapidly inactivated by phosphorylation at Ser 21 (GSK3α) and Ser 9 (GSK3b) in response to insulin [21]. Both GSK3 expression and activity are elevated in muscle and liver tissues of diabetic humans and rodents [22, 23]. Moreover, GSK3 inhibitors improve insulin sensitivity in rodent models of diabetes, alleviating hyperglycemia by decreasing hepatic gluconeogenesis and stimulating glycogen synthesis [24, 25]. Therefore, the present study indicates that elevated expression of PEPCK, G6Pase, and GSK3α may be implicated in etiology of glucose intolerance and type 2 diabetes induced by long-term heavy alcohol consumption.

Accumulating evidence suggests that PEPCK and G6Pase are regulated by 11β-HSD1 and GR via amplification of glucocorticoid action within the tissue [15]. 11β-HSD1, as NADPH-dependent reductase, converts inactive cortisone (11-dehydrocorticosterone in rats) into active cortisol (corticosterone). Enhanced 11β-HSD1 activity results in the production of excess tissue glucocorticoids, which bind and induce local GR activation which is associated with visceral obesity and type 2 diabetes [14, 26]. It has been shown that pharmacological blockade of 11β-HSD1 expression prevents the generation of active glucocorticoids and reduces hepatic GR expression, which in turn results in the suppression of both PEPCK and G6Pase mRNA expression and improvement of insulin resistance in diabetic *db/db* mice and obese Zucker rats [27]. In addition, GR blockade with RU486 attenuated the phenotype of type 2 diabetes through the inhibition of the expression of GR and 11β-HSD1 in the liver [28]. Corticosterone-induced expressions of GR, 11β-HSD1, and PEPCK were also abolished by RU486 [29]. These published data indicate the existence of a positive relationship between GR and 11β-HSD1 in regulation of hepatic gluconeogenic enzymes, implicating GR or 11β-HSD1 as a potential target for the treatment of type 2 diabetes and obesity. The present study showed that 11β-HSD1 and GR protein levels were significantly increased in rats and Hepa 1–6 cells exposed to ethanol, whereas the 11β-HSD1 and GR protein levels were depressed in Hepa 1–6 cells after RU486 treatment. RU486 also reduced the protein expression of PEPCK, G6Pase, and GSK3α, which are regulated by 11β-HSD1 and GR. Therefore, the data suggest that elevated 11β-HSD1 and GR may contribute to the increased expression of PEPCK, G6Pase, and GSK3α in the liver of ethanol-treated rats.

In summary, ethanol-exposed rats have impaired glucose tolerance. The protein expression of enzymes involved in liver gluconeogenesis (PEPCK, G6Pase) and glycogen synthesis (GSK3α) was increased in rats exposed to alcohol in association with an upregulation of 11β-HSD1 and GR. GR blockade with RU486 reversed all these anomalies. The results indicate that elevated 11β-HSD1 and GR, which increase gluconeogenesis and reduce glycogen synthesis, may contribute to the development of glucose intolerance in rats chronically consuming high amounts of alcohol.

Authors' Contribution

Z. Meng and X. Bao contributed equally to this work and are considered co-first authors.

Acknowledgments

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Review Article

Endoplasmic Reticulum Is at the Crossroads of Autophagy, Inflammation, and Apoptosis Signaling Pathways and Participates in the Pathogenesis of Diabetes Mellitus

Jing Su,[1] Lei Zhou,[2] Xiaoxia Kong,[3] Xiaochun Yang,[1] Xiyan Xiang,[1] Yu Zhang,[1] Xiaoning Li,[1] and Liankun Sun[1]

[1] *Department of Pathophysiology, Norman Bethune College of Medicine, Jilin University, Changchun 130021, China*
[2] *Department of Pathology, Affiliated Hospital to Changchun University of Chinese Medicine, Changchun 130021, China*
[3] *Institute of Hypoxia Research, School of Basic Medical Sciences, Wenzhou Medical College, Wenzhou, Zhejiang 325035, China*

Correspondence should be addressed to Liankun Sun; sunlk@jlu.edu.cn

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Diabetes mellitus (DM) is a chronic metabolic disease, and its incidence is growing worldwide. The endoplasmic reticulum (ER) is a central component of cellular functions and is involved in protein folding and trafficking, lipid synthesis, and maintenance of calcium homeostasis. The ER is also a sensor of both intra- and extracellular stress and thus participates in monitoring and maintaining cellular homeostasis. Therefore, the ER is one site of interaction between environmental signals and a cell's biological function. The ER is tightly linked to autophagy, inflammation, and apoptosis, and recent evidence suggests that these processes are related to the pathogenesis of DM and its complications. Thus, the ER has been considered an intersection integrating multiple stress responses and playing an important role in metabolism-related diseases including DM. Here, we review the relationship between the ER and autophagy, inflammation, and apoptosis in DM to better understand the molecular mechanisms of this disease.

1. Introduction

Diabetes mellitus (DM) is a chronic metabolic disease, and its incidence is growing worldwide. Long-term hyperglycemia is the fundamental factor that promotes vascular lesions and dysfunction, leading to a variety of complications of DM [1]. Diabetic complications, such as neuropathy vasculopathy, are the main cause of death or disablement in DM patients [2]. The main purpose of clinical treatments for DM is to control blood glucose and consequently inhibit or alleviate the initiation and progression of complications. However, the control of blood glucose is not easy to achieve [3]. Therefore, a better understanding of the pathogenesis of DM is very important for the development of new treatment strategies.

The endoplasmic reticulum (ER) is an important membranous organelle; its functions include folding and trafficking of protein, lipid synthesis, maintaining calcium homeostasis, and participating in a number of crucial cellular functions [4]. The ER can monitor and maintain cellular homeostasis by acting as a sensor of various changes (stresses) in the intra- and extracellular environment [5]. The ER may therefore provide a platform for interactions between environmental signals and basic cellular biological functions and act as an intersection to integrate multiple stress responses. The interruption of cellular homeostasis can lead to a gradual reduction of organ function, and in turn decreased ability to respond to physiological stress. Recently, a growing body of research has suggested that the ER is involved in the pathogenesis of DM and its complications [6, 7]. Additional research is required to investigate the roles of the ER and its related signaling networks in DM and to thus help develop novel therapeutic strategies.

2. The Unfolded Protein Response and ER Stress

The ER is an important center of multiple cellular processes; it has the ability to regulate synthetic, metabolic, and

adaptive responses to both intra- and extracellular stress and plays a crucial role in maintaining cell homeostasis. When unfolded or misfolded proteins accumulated in the ER lumina, an adaptive response called the unfolded protein response (UPR) occurs [8]. The typical UPR consists of three pathways in eukaryotic cells, which are mediated by three ER membrane-associated proteins: PKR-like eukaryotic initiation factor 2a kinase (PERK), inositol requiring enzyme 1 (IRE1), and activating transcription factor-6 (ATF6). These sensors can monitor changes in the ER lumen and activate downstream signaling pathways. Under stress-free conditions, these sensors are combined with the ER chaperone Bip/GRP78 (glucose regulated protein 78) and exist in their deactivated form [9, 10]. When misfolded proteins accumulate in the ER lumina, UPR sensors detach from GRP78, causing oligomerization and activation of PERK and IRE1 and leading to the activation of downstream signaling pathways [8]. ATF6 is translocated to the Golgi apparatus, where processing by serine protease site-1 protease (S1P) and serine protease site-2 protease (S2P) produces a new active transcription factor [11]. Under ER stress, ATF6 is reduced, and only reduced ATF6 can translocate to the Golgi apparatus, indicating that redox state is one of the factors that determines activation of ATF6 [12]. The UPR can alleviate ER stress by reducing protein synthesis, promoting protein degradation and producing chaperones to assist with protein folding [13]. Excessive or prolonged ER stress can lead to cell death mediated by apoptosis [14].

To date, studies investigating the roles of UPR and ER stress in human diseases have mainly focused on the PERK and IRE1α pathways. Because of the lack of effective research methods and pharmacological tools, the available data regarding the potential role of ATF6 are not sufficient. The adaptability of ER dysfunction can cause UPR activation, and the UPR and ER stress are linked to many different stress signaling pathways [15–17]. This indicates that the ER may be an intersection at which the integration of multiple stress reactions occurs, and it may play an important role in the pathogenesis of chronic metabolic diseases such as type 2 diabetes.

3. ER Stress and Autophagy

Autophagy is a highly conserved and tightly regulated cellular process. Autophagy is a pathway that allows energy/constituent recycling. It also participates in the degradation of misfolded proteins and damaged organelles and facilitates cellular health under various stress conditions including hypoxia, ER stress, or oxidative stress [18–20]. Although the role of autophagy in normal ER function is not established, there are some studies that have shown that autophagy is associated with the ER and maybe an important part of normal ER function [21, 22]. ER stress-induced autophagy plays an important role in maintaining cellular homeostasis through alleviating stress. The ER is related to two major degradation processes in eukaryotic cells: the ubiquitin-proteasome pathway and the autophagy-lysosome pathway [23]. ER stress-induced autophagy can also be used as an alternative degradation mechanism to process misfolded proteins that have accumulated in the ER lumen and that could not be removed by the endoplasmic-reticulum-associated protein degradation (ERAD) pathway. Under over nutrient, high mTOR activity suppresses the mammalian autophagy-initiating kinase Ulk1 by phosphorylating Ulk1 Ser 757 and disrupting the interaction between Ulk1 and AMPK. It suggests that obesity can regulate autophagy through the mTOR signaling pathway [24]. However, under conditions of excessive nutrition, the regulatory mechanisms of autophagy and its effects on insulin action still need further study.

Autophagy plays important roles in metabolic organs, and abnormal autophagy is involved in the pathogenesis of metabolism-related diseases including diabetes and kidney disease [25]. It has been shown that autophagy is essential to islet function and survival and that autophagy deficiency can lead to islet degeneration and reduced insulin secretion [26, 27]. Sequestosome 1 (SQSTM 1/p62) is an important autophagy-related protein. SQSTM 1/p62 deficient mice exhibit metabolic abnormalities and diabetes [28, 29]. Suppression of autophagy can lead to accumulation of reactive oxygen species in the mitochondria, and this may cause initiation of early diabetic nephropathy. Autophagy deficiency in kidneys of diabetic animals can lead to tubule cells being vulnerable to hypoxia and ER stress and can result in progression of diabetic nephropathy [25]. Activation of autophagy may therefore be one therapeutic option for end-stage diabetic nephropathy. Autophagy plays a major role in fatty liver which is directly involved in type II diabetes complications. Autophagy is insufficient in hepatocytes in obesity or fatty liver patients. Its upregulation improves insulin sensitivity and maybe helpful in the treatment of DM [30].

4. ER Stress and Inflammation

Inflammation is involved in the mechanism of metabolism-related diseases such as DM and obesity [31, 32]. Controlling the inflammatory process can therefore be a potential therapeutic target to inhibit the progress of metabolism-related diseases. ER stress and UPR signaling pathways are linked with some of the major inflammation and stress signaling networks including the JNK-AP1 and NF-κB-IKK pathways [33, 34]. In JNK-deficient mice, the expression of proinflammatory cytokines (including TNFα, IL-6, and MCP-1) induced by obesity is suppressed, and this can promote protection from insulin resistance and T2DM (type 2 diabetes mellitus) [35–38]. The IRE1α and PERK pathways of UPR can activate the NF-κB-IKK signaling pathway (inducing the expression of a wide variety of inflammatory mediators) and participate in insulin resistance [39]. Recently, it was shown that the ATF6 pathway is also linked with the NF-κB-IKK pathway, and it has been suggested that specific inflammatory activators can transmit signals through different UPR pathways [40].

The relationship between ER stress and inflammation is not one-way. The activation of inflammatory mediators and cellular stress signaling pathways, such as the JNK and IKK pathways, may have a negative impact on ER function [15, 41]. Notably, exposure to inflammatory cytokines such as TNF-α can induce ER stress, and ER stress itself can cause an increase in the expression of TNF-α or perhaps more general

inflammatory responses [42]. However, the interactions between inflammation and ER stress may be different depending on cell type. Additional research is therefore needed to determine the sensitivity of ER homeostasis to inflammatory signals and the effects of inflammatory stress on ER homeostasis in different cells.

The tight relationship between ER stress and inflammation integrates ER function and metabolic homeostasis and plays a crucial role in obesity, insulin resistance, T2DM, and other metabolic abnormalities [42]. Chronic inflammation can cause ER stress in adipose tissue, and trigger cell damage or death to some degree [3, 43–45]. In metabolic organs, how the ER and its integrated stress signaling system associate with the function of immune cells is unclear [46]. In chronic inflammation, there is evidence that metabolic cells (such as adipocytes and liver cells) can be driven by metabolic hormones and that this can interrupt immune effectors and diffuse inflammatory signaling [47]. However, studies are still needed to clarify the important roles of ER stress and inflammation in metabolism.

5. ER Stress and Apoptosis

If excessive and prolonged ER stress cannot be alleviated by the UPR, functional homeostasis of the ER cannot be reestablished, and this will induce cell death by apoptosis [14]. The PERK pathway activation can induce expression of the proapoptotic transcription factor *chop/gadd153* and activate caspase-12 and JNK signaling pathways, thus leading to apoptosis [48]. IRE1α pathway activation can initiate the proapoptotic Bcl-2 protein and activate Bax and Bak and can also cause tumor necrosis factor receptor-associated factor 2 (TRAF2) and apoptosis signal-regulating kinase 1 (ASK1) recruitment to the cytoplasmic side of the ER membrane, thus leading to apoptotic signaling [49, 50].

As mentioned above, PERK and IRE1α pathway activation can also regulate the NF-κB-IKK signaling pathway by IKK activation or p65 degradation during ER stress. The ATF6 pathway can regulate activity of NF-κB. All of these signaling pathways can trigger apoptosis during excessive or prolonged ER stress. Differences between activation of the three UPR pathways are likely to determine apoptosis [8]. For example, continuous ER stress can reduce IRE1 and ATF6 activity and activate PERK, leading to apoptosis [51]. Similarly, reduction of IRE1 RNA ribonuclease activity can suppress its protective role through producing XBP1 and trigger JNK activation or release proapoptotic mediators [52]. Degradation of mRNA located in the ER by IRE1α may be an important mechanism of ER stress that determines cell death or cell survival [53]. This indicates that IRE1 may be the key "lever" to determine cell fate. The roles of each UPR initiation factor in recognition and response to various types of ER stress are not completely understood. There is also a need for further research to establish how the different UPR pathways function under particular conditions and different cellular environments and to determine whether they participate in different reactions and produce different effects on cells. Although the major function of the ER is protein processing, factors in addition to protein overload can trigger UPR; interruption of ER calcium homeostasis, pathogens or pathogen-related components, or lipids and toxins can also induce ER stress and lead to apoptosis.

Apoptosis is involved in the pathogenesis and progression of DM, and pancreatic beta-cell apoptosis can cause islet degeneration and decrease insulin secretion. In human and experimental kidney diseases such as diabetic nephropathy, hypertensive nephrosclerosis, and glomerulonephritis, glomerular cells including mesangial cells and podocytes can upregulate apoptosis. Prevention of apoptosis may therefore help relieve the progress of DM and its complications.

6. ER Stress in DM

Lipid or inflammatory pathways can trigger insulin resistance, lead to abnormal glucose utilization and blood glucose levels, and promote ER stress. Recent studies have shown that ER stress is involved in obesity, insulin sensitivity, and DM and that the UPR plays an important role in the pathogenesis of these diseases. XBP1-deficient cells or mice are more sensitive to ER stress and insulin resistance because of PERK- and IRE1α-dependent JNK activation. This indicates that there is a causal relationship between sensitivity to ER stress and insulin resistance [54]. In contrast, cells with a higher level of XBP1 tolerate ER stress tolerance and show protective effects against insulin resistance[54]. The ER chaperone oxygen-regulated protein 150 (ORP150), which is activated by the UPR, plays a protective effect on maintaining metabolic homeostasis in mice [55, 56]. ORP150 deficiency in the whole body, particularly the liver, can impair glucose tolerance or reduce insulin receptor signaling by IRS1 phosphorylation [57]. In contrast, in obese or diabetic mice, ORP150 overexpression can lead to increased glucose tolerance and enhanced insulin receptor signaling. Similarly, GRP78 overexpression has beneficial metabolic effects, including reducing liver steatosis and increasing insulin sensitivity in the liver in obese mice [58]. In addition, EIF2α mutant heterozygote mice exhibit ER dysfunction, which leads to obesity and T2DM when fed a high-fat diet [59]. In short, studies based on these animals model provide evidence that ER function is linked to DM.

The effects of ER stress and the UPR on pancreatic islet survival and function play crucial roles in the pancreas. For example, PERK-deficient pancreatic beta cells are more sensitive to apoptosis induced by ER stress. PERK-deficient mice exhibit severe hyperglycemia soon after birth because of a defect in pancreatic proliferation and increased apoptosis [60]. In pancreatic beta cells, suppression of eIF2α phosphorylation can also prompt DM progression which is supported to be secondary to oxidative stress [61]. ER chaperone p58 (IPK) deficiency can promote beta cell dysfunction [62]. In humans, the PERK-eIF2α pathway is very important to islet survival and function. In fact, Wolcott-Rallison syndrome is characterized by a severe beta-cell defect, and its pathogenesis includes PERK dysfunction [63]. In humans, a mutation the *WFS1* gene encoding the ER transmembrane protein wolframin increases the incidence of DM in Wolfram syndrome patients [64].

A better understanding of ER stress in the pathogenesis of DM and its complications may provide novel research directions and therapeutic strategies. Some studies have shown that two chemical chaperones, phenyl butyric acid and tauroursodeoxycholic acid, can suppress ER stress in adipose tissue and liver tissue in obese mice, leading to a decrease in inflammatory signaling and increased insulin receptor signaling. This leads to enhanced circulating insulin sensitivity, normalizes blood glucose, and suppresses the progress of hepatic steatosis [65]. Research based on a lipid-induced ER stress model has shown that ER stress can inhibit apolipoprotein B 100 (apoB100) secretion in liver, which is the major factor promoting hepatic steatosis. Chemical chaperones can also suppress insulin resistance induced by inhibition of apoB100 secretion in liver [66]. The application of these compounds in the treatment of human diseases still has limitations because of the need for very high doses to be effective and because of undesirable pharmacokinetic effects. Further research is therefore needed to clarify how to enhance the ability of endogenous chaperones, promote protein folding, and enhance adaption of the UPR under metabolic stress in active metabolic tissue. This will help to develop new treatment strategies.

Another direction for therapeutic strategies is to reduce ER stress or regulate ER function, including directly targeting mediators of the UPR. Salubrinal is a small molecule that can inhibit eIF2α phosphorylation [67] and suppress cell death induced by ER stress in vivo and in vitro. Recently, a study has shown that salubrinal may activate the IRE1 pathway without causing JNK activation and may have a protective effect on islet cells [68]. Interestingly, some compounds that are currently in clinical trials or that have been used in T2DM treatment, such as PPAR agonists or salicylates, can affect the activity of key ER molecules [69, 70]. One research "hotspot" focuses on whether the therapeutic effects of these compounds are at least partly due to regulation of ER function or of the UPR.

7. Conclusions

In recent decades, many studies have investigated the molecular mechanisms underlying the pathogenesis and progression of DM and complications associated with DM, with the aim of developing novel therapeutic strategies. Yet, the incidence of DM and its complications is still increasing worldwide, and need for development of new therapies targeting is therefore urgent. DM is therefore urgent. As stated above, the ER may be an intersection of integrated multiple stress responses and may be closely related to autophagy, apoptosis, and inflammation. The ER is therefore an attractive potential therapeutic target, and maintaining or improving ER function appropriately may prevent chronic metabolic disease. However, further studies to understand the theoretical and experimental basis of such a potential "organelle treatment" are required before such therapies can be applied.

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Review Article

Adipokines and Hepatic Insulin Resistance

Yu Li,[1] Lin Ding,[1] Waseem Hassan,[1,2] Daoud Abdelkader,[1] and Jing Shang[1]

[1] *National Center for Drug Screening and State Key Laboratory of Natural Medicines, China Pharmaceutical University, Jiangsu Province 210009, China*
[2] *Department of Pharmacy, Bahauddin Zakariya University, Multan 60800, Pakistan*

Correspondence should be addressed to Jing Shang; shangjing21cn@163.com

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Obesity is a major risk factor for insulin resistance and type 2 diabetes. Adipose tissue is now considered to be an active endocrine organ that secretes various adipokines such as adiponectin, leptin, resistin, tumour necrosis factor-α, and interleukin-6. Recent studies have shown that these factors might provide a molecular link between increased adiposity and impaired insulin sensitivity. Since hepatic insulin resistance plays the key role in the whole body insulin resistance, clarification of the regulatory processes about hepatic insulin resistance by adipokines in rodents and human would seem essential in order to understand the mechanism of type 2 diabetes and for developing novel therapeutic strategies to treat it.

1. Introduction

In many developed and developing countries, obesity has reached epidemic proportions, resulting in an increasing prevalence of type 2 diabetes characterized by insulin resistance of peripheral tissues such as liver, muscle, and fat which cannot be overcome by hypersecretion of pancreatic beta cells [1]. One survey conducted in 2000 revealed that more than 150 million people in the world suffered from type 2 diabetes [2] and 80% of these cases were related to obesity. Because various studies have demonstrated that hepatic insulin resistance plays a central role in the development of type 2 diabetes and obesity is centrally involved in increasing the clinical risk of diabetes, visceral adipose tissue is now thought to provide a link between obesity and hepatic insulin resistance.

Adipose tissue was traditionally regarded as a passive energy reservoir. However, since the discovery of leptin and subsequent identification of other adipose tissue-derived cytokines (e.g., adiponectin and resistin) in the last two decades [3], it became clear that adipose tissue is an active endocrine organ. Obese adipose tissue also secretes various inflammatory cytokines, such as interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) [4]. All of these cytokines, termed adipokines, act in an autocrine, paracrine, or endocrine fashion to control various metabolic functions. Some of these adipokines have been implicated in the development of hepatic insulin resistance. Indeed, they may act locally or distally to alter insulin sensitivity in insulin-targeted organs such as liver which is also discussed in detail by Marra and Bertolani [5] previously or may act through neuroendocrine, autonomic, or immune pathways. For example, activation of proinflammatory pathways in adipose tissue is known to interfere with insulin signaling and induce hepatic insulin resistance [6]. Although, the role of adipocytokines on insulin resistance has been comprehensively elucidated elsewhere [7, 8], but recently specialized review on their actions in hepatic insulin resistance is missing. In this paper, we focus on the role of a series of adipokines and discuss how they influence hepatic insulin sensitivity.

2. Adipokines and Hepatic Insulin Resistance

2.1. Adiponectin. Since the identification of adiponectin as a protein exclusively produced from adipocytes exclusively by Scherer et al. in 1995 [9], it has grabbed much attention by scientific communities, primarily due to its inverse relationship with hepatic insulin resistance. It was first named as adipocytes complement related protein of 30 kDa (ACRP30) on the basis of structural similarity with the complement C1q fraction [9]. At the same time other research groups

also termed it as AdipoQ, adipose most abundant gene transcript 1 (apM1) or (gelatin-binding protein 28) GBP28, in both mice and humans [10]. Following the characterization of its genomic organization, comprising three exons and its 3q27 localization in 1999 [11] the protein was named adiponectin [12]. In 2004, the gene was named adipocytes C1q and collagen-domain-containing (ACDC), but actual nomenclature given by the Human Genome Organization (HUGO) nomenclature is now ADIPOQ [13].

The adipokine adiponectin is greatly expressed in human serum where it accounts for 0.01% of total plasma protein and is predominantly secreted by adipose tissue [14] (reports of secretion in differentiating preadipocytes [15] and placenta [16]) in inverse proportion to the body mass index [17]. A unique feature of the structure of adiponectin is its ability to assemble into several characteristic oligomeric isoforms, including trimers (low molecular weight (LMW)), hexamers (middle molecular weight (MMW)), and the oligomeric complexes comprising 18 protomers or above (high molecular weight (HMW)) [18]. Adiponectin presents predominantly in the circulation in the three oligomeric complexes cited previously [19]. Trimeric adiponectin is the basic unit block of adiponectin. Two LMW adiponectin molecules linked by disulfide bonds to form hexameric adiponectin [18]. Adiponectin may also form hetero-oligomers with additional members of the C1q/TNF-related protein (CTRP) family such as the CTRP9 [20].

The role of adiponectin in hepatic insulin resistance has evolved into a major factor and seen by many as therapeutic option now. The generalized effects of adiponectin on insulin resistance can be cited elsewhere [21, 22]. In this portion we will review the major studies describing the role of adiponectin and its receptors [23] in hepatic insulin resistance.

Hepatic insulin resistance is a hall mark for the spectrum of many diseases, and it is an independent predictor for metabolic disorders. Recent evidence suggests that visceral adipose tissue is a metabolic and inflammatory organ that signals and modulates the action and metabolism of the brain, liver, muscle, and cardiovascular system [24, 25]. The hormone plays a role in the attenuation of the hepatic metabolic disorders that may result in type 2 diabetes [26], insulin resistance, obesity [27], and nonalcoholic fatty liver disease (NAFLD) [28].

Recent data has exhibited more specification as most of the studies have implicated HMW oligomers in attenuation of hepatic insulin resistance. For instance, the synthesis of the HMW oligomers is necessary to mediate the insulin sensitizing effects of adiponectin on the suppression of hepatic gluconeogenesis in primary rat hepatocytes [29]. Moreover, HMW oligomers of adiponectin potently blunted hyperglycemia in diabetic mice through the inhibition of hepatic glucose production [30]. Further, acute injection of recombinant adiponectin enriched with the HMW oligomers resulted in a marked activation of AMP-activated kinase (AMPK) in the liver, while chronic infusion with this protein leads to prolonged alleviation of hyperglycemia and insulin resistance in *db/db* diabetic mice [31]. Animal studies are matched to the clinical data exhibiting that the ratio of HMW/total adiponectin correlates closely with hepatic insulin sensitivity [32]. The role of the HMW oligomer as a predominant active form of adiponectin mediating its hepatic actions is also supported by two recent independent reports demonstrating that the insulin-sensitizing effects of the peroxisome proliferator-activated receptor gamma (PPAR-γ) agonist thiazolidinediones were diminished in *ob/ob* obese mice with the targeted mutation of the adiponectin gene [33]. Notably, treatment with thiazolidinediones, which is an insulin sensitizer caused a selective increase of the HMW oligomeric adiponectin [32]. Pioglitazone, another insulin sensitizer, has remarkably increased the HMW adiponectin, which correlated greatly with the increased hepatic insulin action [34].

Plethora of animal data has been published describing the role of adiponectin in hepatic insulin resistance. Different animal models have supported this supposition widely. For example, a high scale study of adiponectin function in animal models of obesity, insulin resistance, and type 2 diabetes provided strong evidence that adiponectin promotes insulin sensitivity in muscle and in the liver [35]. Further, most of the adiponectin knockout mice were more insulin resistant than controls, although to different degrees, and this factor has been associated with hepatic insulin resistance [36].

Moreover, adiponectin-deficient mice showed mild insulin resistance in the liver with administration of standard diet [36]. Similar evidences were extracted on adiponectin receptor (AdipoR) studies, as adipoR1 and adipoR2 knockout mice exhibit mild insulin resistance [37]. Further, AdipoR2 knockout mice showed reduced diet-induced insulin resistance but promoted type 2 diabetes [38]. In another study, secretion of wild type adiponectin has been employed [39] and caused insulin sensitization when expressed in either liver [39] or adipose tissue [40]. Interestingly, infusion of adiponectin inhibited both the expression of hepatic gluconeogenic enzymes and the rate of endogenous glucose production by the liver. The latter was dependent on insulin, because adiponectin alone had no significant effect on glucose output in cultured hepatocytes. Obviously, both wild-type and type 2 diabetic mice were shown to reduce the expression of gluconeogenic enzymes and elevated phosphorylation of hepatic AMP-activated protein kinase (AMPK), and the effects were attributes to adiponectin due to its circulating levels [41]. Indeed AMPK has currently established itself as a target for adiponectin, and its phosphorylation has been linked with the adiponectin level [42]. It is worth mentioning here that most of the adiponectin effects on liver has been attributed to either AMPK activation or nuclear receptor involvement, and this activation is mediated by AdipoR2, but a recent ground breaking study refuted this as an only view and put forward new dimensions in future adiponectin research. Motoharu and his group have shown that adiponectin effects on hepatic system can be independent of AdipoR2 and can also be mediated by inflammatory cells [43].

Clinical studies have described the similar evidences regarding the role of adiponectin in liver insulin resistance. Instantly, adiponectin levels are associated, in healthy humans, with plasma concentrations of various liver function

indices [44]. Further, a clinical study showed that plasma level of adiponectin was closely linked with hepatic lipids and insulin resistance in patients administered with pioglitazone [45]. Another clinical study supported the similar view and showed that increased adiponectin level could help restore the hepatic insulin resistance in severely obese women [46].

In conclusion, adiponectin is a promising target for hepatic insulin resistance and resultantly metabolic disorder, but further clinical research is required to translate similar impacts in humans keeping in mind the pleiotropic nature of this hormone.

2.2. Leptin. In 1994, ob gene was first cloned on mice chromosome 6 [47]. A year later, Halaas found that leptin was the gene product of ob in colon bacillus [48]. Leptin is a 16-KDa hormone secreted by adipocytes which plays a key role in the regulation of food intake, energy metabolism, saccharide, lipid metabolism, and so forth. The levels of leptin in adipose tissue and plasma are dependent on the amount of energy stored like fat as well as the status of energy balance. Therefore, leptin levels are higher in obese individuals and increase with overfeeding [49, 50].

Lots of studies reported that a strong positive correlation exists between the concentration of leptin and insulin sensitivity and obesity [51–54], but the anti-insulin resistance effect of leptin, independently of its activity on weight control, was also reported [55, 56]. The mechanisms that explain the direct role of leptin in hepatic insulin resistance are not well understood. Studies in animal model showed that exogenous leptin administration leads to a dramatic improvement in insulin resistance that is independent of decreased caloric intake [57]. Clinical studies showed that chronic leptin treatment ameliorates insulin-stimulated hepatic and peripheral glucose metabolism in severely insulin-resistant lipodystrophic patients [58]. A study showed that in leptin receptor-deficient Koletsky rats, adenovirally induced expression of leptin receptors in the area of the hypothalamic arcuate nucleus improved peripheral insulin sensitivity via enhancement of suppression of hepatic glucose production, with no change of insulin-stimulated glucose uptake or disposal, and leptin regulated hepatic insulin sensitivity via phosphatidylinositol-3-OH kinase (PI3K) signaling in the liver of this animal model [59].

In summary, leptin acts as an important target to improve the hepatic sensitive to insulin, and the precise mechanisms about leptin underlying hepatic insulin resistance should be investigated by experiments *in vivo* and *in vitro*.

2.3. Resistin. Resistin is a member of a family of cysteine-rich proteins referred to as resistin-like molecules, termed RELMs, which include RELM-α, RELM-β, and the recently discovered RELM-γ [60]. It was discovered in 2001 by Dr Mitchell A. Lazar [61] and was called resistin, since mice injected with resistin exhibited insulin resistance. Crystallographic studies of resistin have determined its complex hexameric structure. Resistin is proved to circulate in two distinct assembly states, likely corresponding to hexamers and trimers. Infusion of an intertrimer disulfide bonds-lacking resistin showed more potent effects on hepatic insulin sensitivity than those observed with wild-type resistin [62]. This result suggested an activation effect of different intertrimer disulfide bonds and further suggested a potential target site for the receptor interaction.

In rodents, resistin primarily expressed in and secreted from mature adipocytes, with some expression in pancreatic islets and portions of the pituitary and hypothalamus. However, resistin is expressed primarily by macrophages and seems to be involved in the recruitment of other immune cells and the secretion of proinflammatory factors in humans [63]. The diverse expression and regulatory patterns may be explained by the evidence that human and mouse resistins have diverse genomic organizations [64]. Moreover, at the protein level, human resistin is only 55% identical to its murine counterpart.

A study in rodents showed that the infusion of resistin rapidly induced severe hepatic insulin resistance, resulting in a reduced insulin-mediated suppression of gluconeogenesis and increased glycogenolysis [65]. Consistent with these reports, resistin induced insulin resistance with a robust decrease in insulin-stimulated phosphorylation of Akt and glycogen synthase kinase3 (GSK3) human liver cell line HepG2 cells [66], pointing to a specific role of resistin in the initiation of hepatic insulin resistance. Furthermore, mice treated with an antiresistin IgG [67] or expressing a dominant negative resistin [68] exhibit low fasted blood glucose levels due to reduced hepatic glucose production.

In a word, resistin could induce hepatic insulin resistance by inhibiting the phosphorylation of Akt and GSK3, and its effect on insulin sensitivity is opposite to those reported for the adipocyte-secreted hormone adiponectin, which increases insulin sensitivity of the same liver-specific functions.

2.4. IL-6. IL-6 is secreted by many cell types, including immune cells, fibroblasts, endothelial cells, myocytes, and a variety of endocrine cells [69]. Adipose tissue contributes to 10–35% of circulating IL-6 [70] in resting, healthy humans, and the production is greater in obese subjects [71]. It is a multifunctional cytokine that has been well known for its anti-inflammatory and proinflammatory effect in immune responses. IL-6 signals through a cell-surface type I cytokine receptor complex consisting of the specific receptor subunit IL-6 receptor (IL-6R) and the signal-transducing component glycoprotein 130 (gp130), which is the common signal transducer for several cytokines including leukemia inhibitory factor (LIF), ciliary neurotropic factor, oncostatin M, IL-11, and cardiotrophin-1 [72]. In contrast, the expression of IL-6R is restricted to certain tissues. IL-6 interacts with its receptor IL-6R and gp130 protein to form a complex that activates the receptor IL-6R.

The inflammatory regulator IL-6 has also emerged as a factor that is implicated in hepatic insulin resistance. However, the role of IL-6 in the etiology of insulin resistance is not fully understood. Impaired insulin receptor signaling and insulin-dependent glycogen synthesis were found in HepG2 cells and primary mouse hepatocytes acutely pretreated with IL-6 [73]. IL-6 caused reduced insulin signal transduction in the liver of mice [74]. These results seemed to be consistent

with the hypothesis that IL-6 may have a negative effect on insulin resistance. But in healthy humans under the basal condition, treatment by recombinant human IL-6 (rhIL-6) at a physiological concentration neither impaired the whole body glucose disposal nor increased endogenous glucose production [75]. In patients with type 2 diabetes, splanchnic glucose output did not increase with acute infusion of rhIL-6 [76], while glucose disposal was not impaired, suggesting that IL-6 might have favorable action on insulin action.

IL-6 mediates insulin sensitivity through many distinct mechanisms. Of note, there is a significant correlation between the level of IL-6 in adipose tissue and several metabolic parameters such as fasting plasma glucose, basal and insulin-stimulated glucose transport, and whole-body glucose disposal [77]. Thus, IL-6 may play an important role in insulin action in these subjects. Moreover, IL-6 mediated suppressor of cytokine signaling (SOCS-3) pathway in liver, leading to impairment of insulin actions [78].

To sum up, the effect of IL-6 on hepatic insulin sensitivity is uncertain; it appeared to be determined by whether it is present acutely or chronically; the latter is the setting associated with insulin resistance.

2.5. TNF-α. TNF-α is a proinflammatory cytokine produced by various types of cells, mainly but not only inflammatory cells like macrophages and lymphocytes. The release of TNF-α in noninflammatory cells has also been reported, at lesser degrees. Since the first recognized sequencing of TNF-α in 1984, it has established itself as a prime proinflammatory cytokine [79], making it the subject of continuous pondering by researchers. It is now clear that TNF-α is involved in array of pathological conditions like obesity, congestive heart failure, inflammations, and insulin resistance [80], nevertheless the exact role and degree of its effects are still not clearly understood.

TNF-α has been proposed as a link between adiposity and the development of insulin resistance [81], because the majority of type 2 diabetic subjects are obese [82]. Elevated levels of TNF-α have been observed in obese and insulin resistance humans and animals [83]. TNF-α-mediated pathway interference has shown protection in diet induced animal models of obesity and metabolic syndrome [84]. A clinical study has observed improvement in the insulin sensitivity under anti-TNF-α therapy [85]. Anti-TNF-α therapy has also shown to attenuate in insulin resistance in animal studies involving fructose-red rats [86]. Further, TNF-α expression is increased in adipose tissue in obese rodents and humans [87], and reducing TNF-α signaling either by knocking TNF-α out or by infusing blocking antibodies can reduce insulin resistance in obese rodents [88]. More recent clinical evidence confirms the proportional rise of TNF-α and IR with parallel increase in body mass index (BMI) factor [89]. A recent cohort study on nondiabetic patients with insulin resistance has shown concomitant decrease in TNF-α level accompanied by an improvement in insulin resistance after administration of rosiglitazone [90]. Another reported study has observed the direct dose-dependant effects of TNF-α and higher doses linked directly to insulin sensitivity [91]. Moreover, insulin stimulating effects on glycemic levels and glucose uptake have been reversed by the administration of TNF-α [92]. A clinical trial which consists of 15 women with normal glucose tolerance and developed gestational diabetes mellitus has proved TNF-α as the sole biomarker and predictor for insulin resistance during pregnancy [93]. In one rodent model, obese mice lacking either TNF-α or its receptors showed protection against developing insulin resistance [94]. Liver resident macrophages (Kupffer cells) in human body are the primary source of TNF-α; diet induced macrophages which subsequently release the TNF-α are found to be involved in insulin resistance in paracrine signaling.

TNF-α is reported to demonstrate wide range of effects in pathogenesis of insulin resistance. The precise signaling pathway is, however, not clear yet even after scores of studies and scientific data put forward on this particular subject. However, the most attention grabbing signaling pathway in liver supported by numerous evidences is through the phosphorylation of the insulin receptor substrate-1 (IRS-1) protein on serine residues. This could prevent its interaction with the insulin receptor beta subunit and stop the insulin signaling pathway [95–97]. However, the precise role of TNF-α in human insulin signaling requires extensive scientific investigation in future to clear its signaling pathway.

Conversely, numerous clinical studies have not been able to support similar findings and failed to prove its role in the relationship between type 2 diabetes coexisting with insulin resistance [98, 99]. TNF-α antibody infusion in humans was shown not to alter insulin sensitivity, fueling lingering uncertainty about the biological relevance of this pathway in human insulin resistance states [100].

Conclusively, TNF-α role in modulating insulin resistance with no doubt varies from species to species and depends on animal models adopted for particular study. Further, due to its release by various types of cell types involving both inflammatory and noninflammatory cells which then can be involved in insulin resistance pathogenesis both by autocrine or paracrine manner, TNF-α is the subject of uncertainty regarding its precise role in insulin resistance. Without doubt, further scientific work is required in order to unravel its exactness in insulin resistance.

3. Summary

During the last couple of years it has been shown that adipokines play an important role in physiology and pathophysiology of insulin sensitivity. Our review summarizes recent findings regarding the relationship between adipocytokines and hepatic insulin resistance (Figure 1). The mechanisms by which adipocytokines promote or relieve insulin resistance are complex, and our understanding is yet incomplete. According to most of the recent studies, it seems that excessive adipose tissue may be detrimental partially through secretion of the following cytokines: TNF-α, IL-6, and resistin. In contrast, the presence of adipose tissue is vital in the prevention of hepatic insulin resistance, at least in part, via secretion of the following cytokines: leptin and adiponectin. Indeed, adipocytokines are major regulators of hepatic insulin sensitivity potentially linking

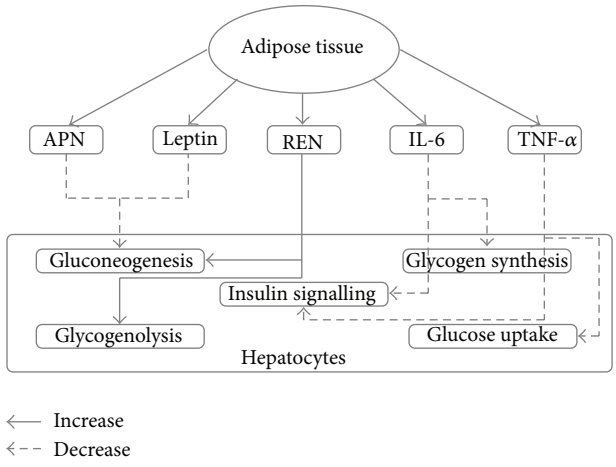

Figure 1: The effects of adipokines on hepatic glucose metabolism and insulin signalling. Both of adiponectin (APN) and leptin can decrease hepatic gluconeogenesis. Resistin (REN) can increase hepatic gluconeogenesis and glycogenolysis. Moreover, interleukin-6 (IL-6) can decrease glycogen synthesis, and tumor necrosis factor α (TNF-α) can decrease glucose uptake in liver. Both of them can block hepatic insulin signalling by interfection of insulin receptor signalling and insulin signal transduction.

insulin resistance and obesity. Further work is needed to clearly determine how they regulate the insulin signaling in hepatocytes and influence insulin sensitivity in other tissues (e.g., muscle and adipose tissue) in rodents and human and their contribution to glucose homeostasis in diabetes.

Authors' Contribution

Yu Li and Lin Ding equally contributed to the project.

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Research Article

Chronic Caloric Restriction and Exercise Improve Metabolic Conditions of Dietary-Induced Obese Mice in Autophagy Correlated Manner without Involving AMPK

Mingxia Cui,[1] Han Yu,[2] Jinli Wang,[3] Junjie Gao,[4] and Ji Li[3]

[1] *Department of Pharmacology, Key Laboratory of Preclinical Study for New Drugs of Gansu Province, School of Basic Medical Science, Lanzhou University, Lanzhou 730000, China*
[2] *Neuroscience Program, Department of Biochemistry, University at Buffalo, Buffalo, NY 14214, USA*
[3] *Department of Pharmacology and Toxicology, University at Buffalo, Buffalo, NY 14214, USA*
[4] *Department of Cardiology, Shuguang Hospital Affiliated to Shanghai University of Traditional Chinese Medicine, Shanghai 201203, China*

Correspondence should be addressed to Ji Li; jli23@buffalo.edu

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Aim. To investigate the role of AMPK activation and autophagy in mediating the beneficial effects of exercise and caloric restriction in obesity. *Methods*. Dietary-induced obesity mice were made and divided into 5 groups; one additional group of normal mice serves as control. Mice in each group received different combinations of interventions including low fat diet, caloric restriction, and exercise. Then their metabolic conditions were assessed by measuring serum glucose and insulin, serum lipids, and liver function. AMPK phosphorylation and autophagy activity were detected by western blotting. *Results*. Obese mice models were successfully induced by high fat diet. Caloric restriction consistently improved the metabolic conditions of the obese mice, and the effects are more prominent than the mice that received only exercise. Also, caloric restriction, exercise, and low fat diet showed a synergistic effect in the improvement of metabolic conditions. Western blotting results showed that this improvement was not related with the activation of AMPK in liver, skeletal muscle, or heart but correlates well with the autophagy activity. *Conclusion*. Caloric restriction has more prominent beneficial effects than exercise in dietary-induced obese mice. These effects are correlated with the autophagy activity and may be independent of AMPK activation.

1. Introduction

Caloric restriction (CR) and exercise have been considered to have beneficial effects on human health, including reducing the risks for the development of diabetes, cardiovascular disease, and cancer [1, 2]. Studies have shown that CR and exercise can improve the metabolic conditions in obesity, but the underlying mechanism is still unclear. The AMP-activated protein kinase (AMPK), as an important energy sensor, can be activated by the increased AMP/ATP ratio or ADP in energy deprival state [3]. AMPK activation plays important roles in adjusting the metabolic pathways to restore the ATP level in both short-term and long-term manner [4]. Because of this activity, it has been considered as a potential mediator of the effects of CR and exercise. However, whether AMPK activation is induced in chronic CR or exercise is still an unresolved issue due to distinct observations on the AMPK activity after long-term CR or exercise in mouse [5].

Autophagy is an intracellular recycling pathway that functions during basal conditions but can be induced under stress such as starvation [6]. Recent findings established the relationship between insulin resistance in obesity and the decreased autophagy activity in liver, and restoration of Atg7 was shown to enhance the systemic glucose tolerance in mice [7]. Further, it has been shown that Bcl2-regulated autophagy is indispensable in mediating the improvement of glucose homeostasis by either long- or short-term exercise [8]. Since AMPK has already been known to participate

Table 1

Group number	Mice	Treatment
1	Normal	
2	Dietary-induced obesity (DIO)	
3	DIO	Low fat diet with CR
4	DIO	High fat diet with CR
5	DIO	High fat diet with exercise
6	DIO	Low fat diet with CR and exercise

in regulating autophagy activity by activating Ulk1 through direct phosphorylation [9], we wonder if chronic CR and exercise can induce autophagy in obese mice and if the improvement in metabolic conditions is correlated with the autophagy activity. Further, we also sought to investigate if there is any relationship between induced autophagy and AMPK activity in obese mice.

To study the relationship between the chronic CR, exercise, and autophagy and whether AMPK activation is involved in this process, we used high fat diet to induce the obese mice models, and then we investigated the effects of different intervention on the improvement of mice metabolic conditions. Then, we examined the level of LC3 proteins and AMPK phosphorylation to investigate the relationship between metabolic improvement and AMPK activation, as well as the relationship with autophagy activity.

2. Materials and Methods

2.1. Mice Models. Studies were conducted on 6-week-old male C57 mice, which were fed with high fat diet containing 58% fat (kcal%, total calorie 5.56 kcal/g). Mice in the control group were fed with normal 5% fat diet (kcal%, total calorie 3.4 kcal/g). Six mice, from the experimental and the control group, respectively, were taken and measured for body weight and 24-hour calorie intake every other week.

2.2. Intervention of CR and/or Exercise. The first two weeks were adaption period. High fat diet was switched to low fat diet, and the running speed of the exercise groups was 8 m/min, 10 min per day, and 7 days a week. The following 6 weeks were therapeutic period. The calorie intake of CR group is around 70% of that of the model control group, a level that has been reported to effectively protect against high fat diet induced obesity in mice [10]. The running speed was gradually increased from 8 to 22 m/min within one week, 30 min/day, and 5 days/week, as described by Reznick et al., which is considered as sufficient to increase the AMP/ATP level and activate AMPK [11]. The mice were grouped as in Table 1.

2.3. Adipocytokines, Serum Glucose, and Insulin. Serum glucose levels were tested by using Glucose (HK) Assay Kit (Sigma-Aldrich). Adipocytokines ad insulin levels were assessed by ELISA.

2.4. Serum Lipids. Triglycerides levels were tested by Triglyceride (GPO Trinder) Reagent A kit. To quantitatively determine the HDL cholesterol in serum, Wako HDL-Cholesterol E assay was used by phosphotungstate-magnesium salt precipitation.

2.5. Liver Function. ALT and AST tests kits (American Screening Corporation) were used to evaluate liver function of the mice. Standard HE staining was used to evaluate the morphological changes of livers.

2.6. Detection of p-AMPK Phosphorylation and LC3 Activity. Liver, skeletal muscle, and heart homogenates were resolved by SDS-PAGE, and proteins were transferred onto polyvinylidene difluoride membranes. Rabbit polyclonal antibodies against phosphorylated AMPK, total AMPK, and LC3 and anti-rabbit secondary antibodies were purchased from Cell Signaling. LC3-II/LC3-I is used as an index of autophagy activity.

2.7. Statistical Analysis. Values are expressed as means ± SE. Data were analyzed by a two-tailed, independent Student's t-test. P values less than 0.05 were considered statistically significant.

3. Results

3.1. Features of Dietary-Induced Obese Mice

3.1.1. Body Weight, Caloric Intake, and Organs Weight. The calorie intake of the DIO mice was consistently higher than that of the control group, and the body weight also increased more rapidly. The weight of control group became stable at week 10, while that of the DIO group continued increasing even at the end of this study. Significant difference exists in the weight, liver, mesenteric fat, postperitoneal fat, and the total visceral fat between the two groups. The weight of the heart also had a significant increase, while no obvious difference was observed in the weight of skeletal muscle. Similarly, the coefficient (organ weight/100 g body weight) of liver, mesenteric fat, postperitoneal fat, and the total visceral fat, increased as well, but that of heart and skeletal muscle had decreased significantly.

3.1.2. Serum Biochemical Indices. By the end of this study, the DIO mice had exhibited hyperinsulinemia, insulin resistance, and increase in cholesterol, triglycerol, HDL-cholesterol, and LDL-cholesterol. Meanwhile, ALT and AST in the serum also increased. These results showed the obese mice models were successfully induced by using high fat diet.

3.2. Effects of Intervention

3.2.1. Body Weight and Caloric Intake. The intake of the Groups 3 and 6, whose diet was switched from high fat to low fat, has decreased at the initial stage. After two-week

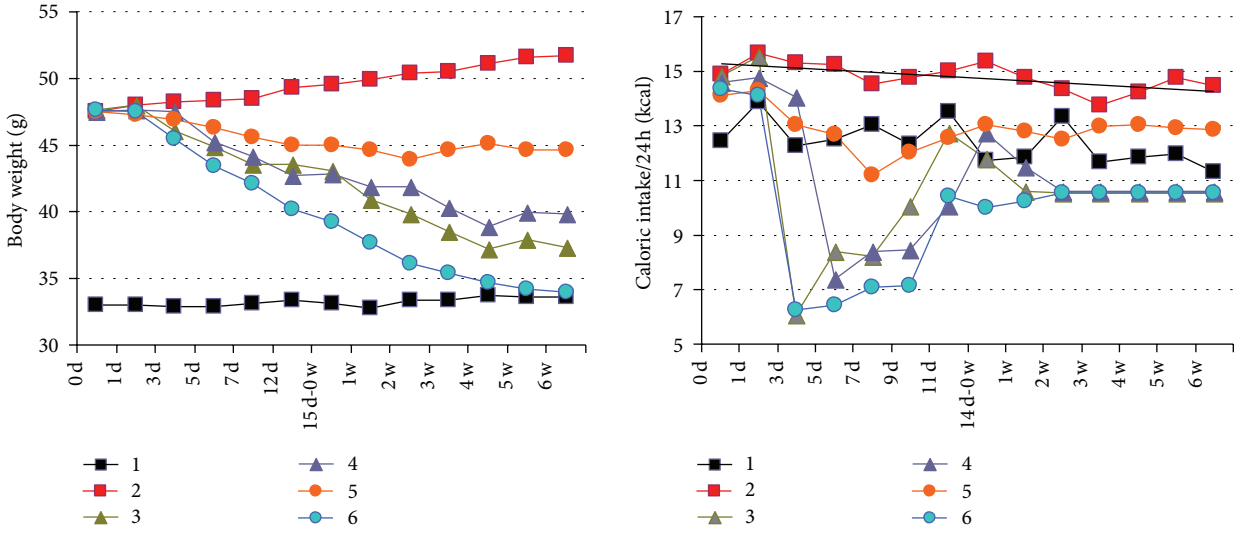

Figure 1: Change in body weight and calorie intake.

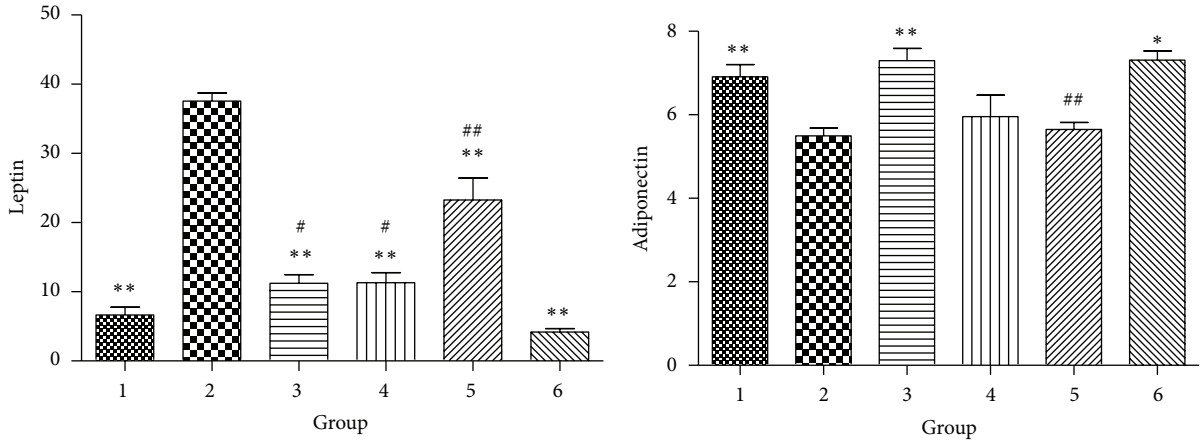

Figure 2: Levels of lectin and adiponectin ($^{*}P < 0.05$ compared with Group 2. $^{**}P < 0.01$ compared with Group 2; $^{\#}P < 0.05$ compared with Group 1. $^{\#\#}P < 0.01$ compared with Group 1).

adaption, the intake of these two groups has gradually recovered and got stable. The calorie intake of the exercise group with high fat diet was significantly lower than that of Group 2. At the end of this experiment, the body weights of Groups 3~6 were all significantly lower compared with Group 2 (Figure 1).

3.2.2. Adipocytokines. The level of leptin, which is an appetite-inhibiting adipokine, in DIO mice significantly decreased after CR and/or exercise, and the level of leptin decreased to a similar extent in the CR treated groups regardless of HFD or LFD. Exercise combined with CR and low fat diet further reduced the leptin level. The level of adiponectin was significantly reduced in DIO mice but was recovered in the groups treated with LFD and CR, regardless of exercise. However, in HFD groups neither CR nor exercise had a significant effect on adiponectin level (Figure 2).

3.2.3. Serum Glucose and Insulin. Serum glucose slightly increased in DIO mice and reduced to a small extent in mice with all the treatments, but most of the differences were not statistically significant. Only the group of HFD plus CR showed a significant improvement. The insulin level also increased in DIO mice, but to a higher degree, suggesting insulin resistance was induced in the obese model and can be recovered by CR or CR plus exercise. Interestingly, however, LFD with CR did not improve the insulin resistance conditions as HFD plus CR did. This plausible result was probably caused by the large variation in this group's insulin level, since the average actually exhibited a decreasing trend when compared with DIO group (Figure 3).

3.2.4. Serum Lipids. Hyperlipidemia was found in the DIO mice. Among the treatments, CR alone can significantly decrease the cholesterol and LDL-cholesterol levels. Further,

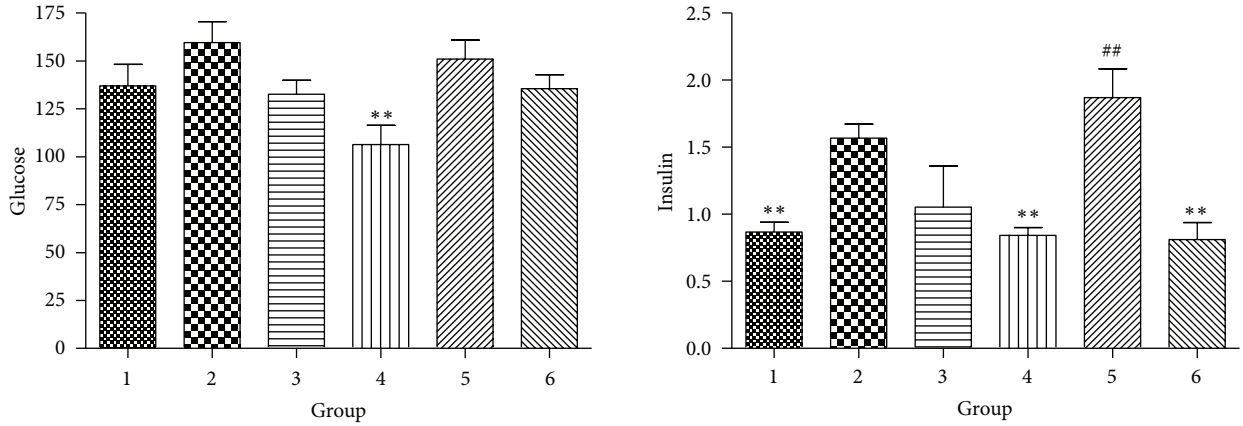

Figure 3: Levels of serum glucose and insulin ($^{*}P < 0.05$ compared with Group 2. $^{**}P < 0.01$ compared with Group 2; $^{\#}P < 0.05$ compared with Group 1. $^{\#\#}P < 0.01$ compared with Group 1).

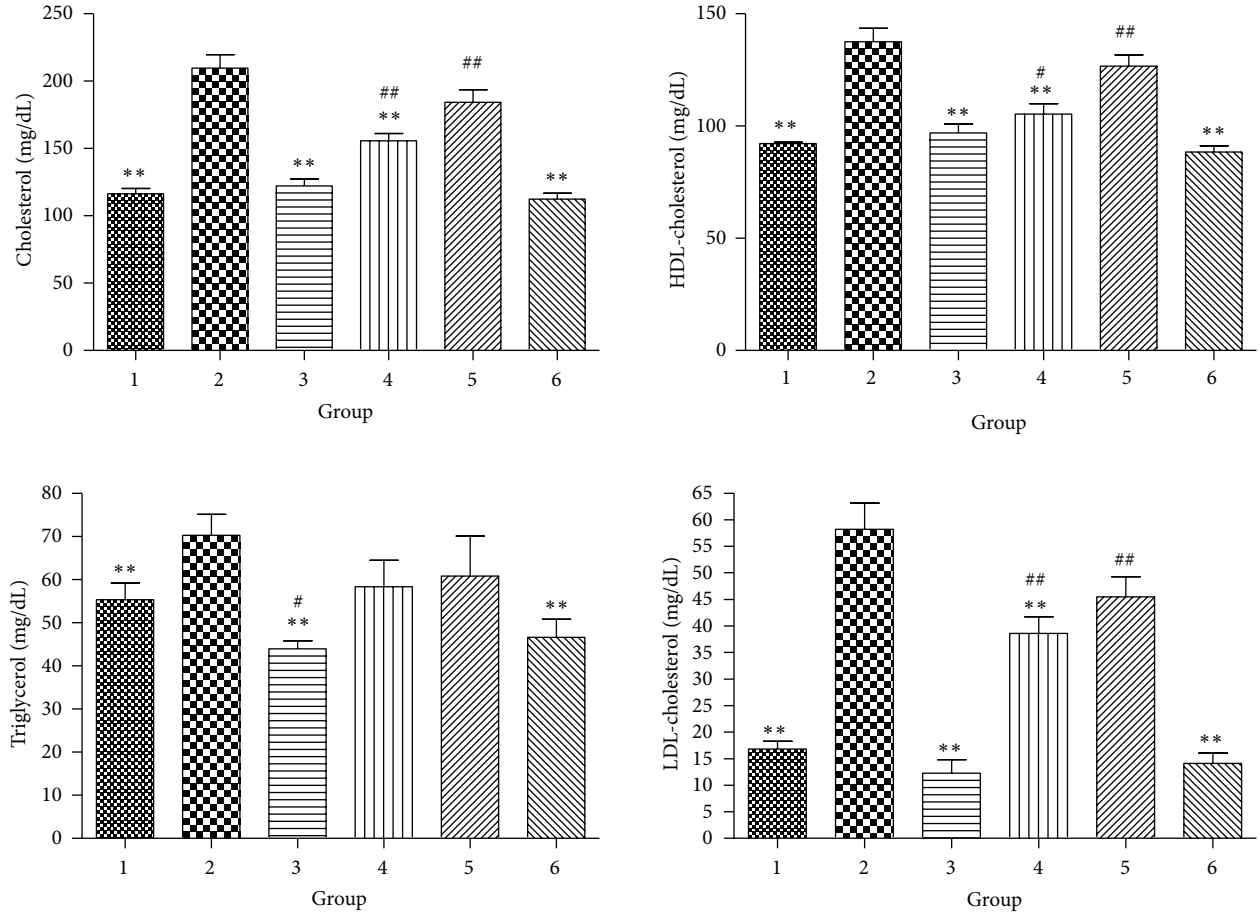

Figure 4: Levels of serum lipids ($^{*}P < 0.05$ compared with Group 2. $^{**}P < 0.01$ compared with Group 2; $^{\#}P < 0.05$ compared with Group 1. $^{\#\#}P < 0.01$ compared with Group 1).

when combined with LFD and/or exercise, the effects became even more prominent. LFD and CR can reduce the level of triglyceride either with or without exercise training (Figure 4).

3.2.5. Liver Function. Alteration in AST and ALT levels has been shown to be related with metabolic conditions and directly associated with insulin resistance [12]. In addition, these markers can be also used to assess the extents of liver injury in metabolic disorders. Therefore, we further examined their serum levels. While both AST and ALT levels increased in DIO mice, they can be reduced to normal level by either CR or exercise. In consistence with the hyperlipidemia conditions, different extents of hepatic steatosis were found

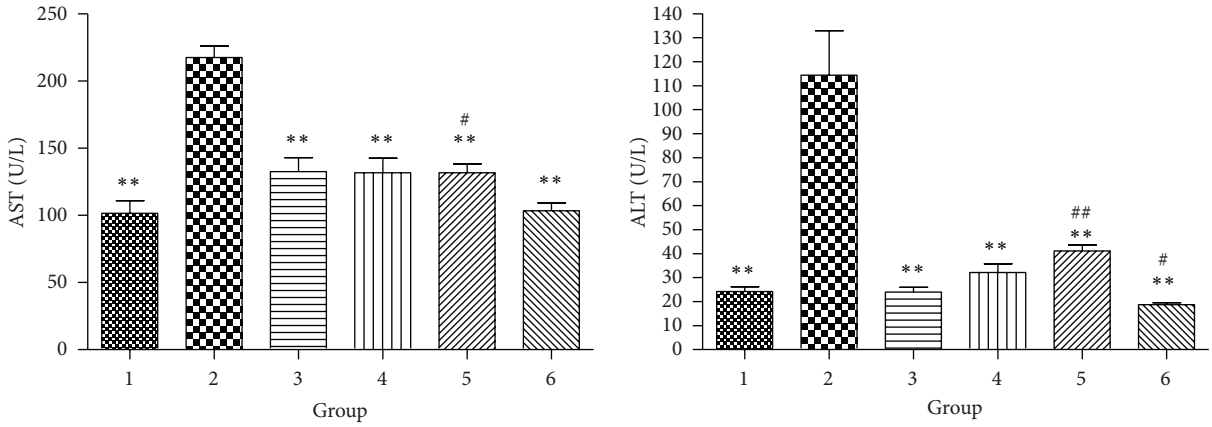

Figure 5: Levels of AST and ALT in serum ($^{*}P < 0.05$ compared with Group 2. $^{**}P < 0.01$ compared with Group 2; $^{\#}P < 0.05$ compared with Group 1. $^{\#\#}P < 0.01$ compared with Group 1).

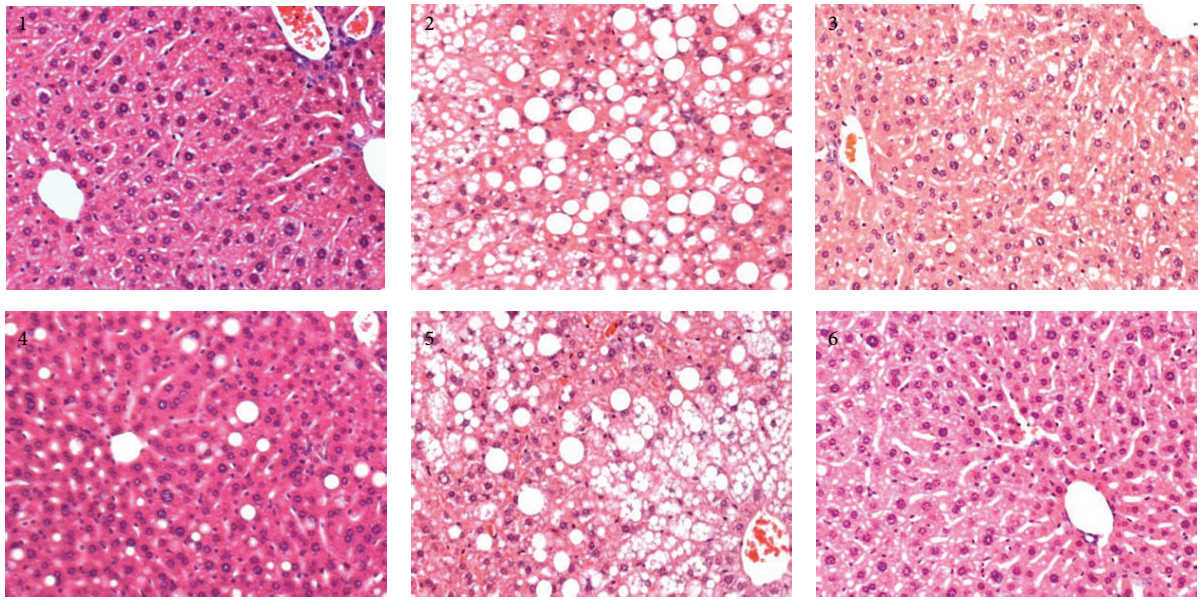

Figure 6: Representative images of H&E staining of liver sections (20x). The numbers on the upper-left corner indicate the group. The liver in Group 1 is generally normal, while obvious hepatic steatosis can be found in Group 2. Hepatic steatosis is much relieved in Group 3, 4, and 6, and that of Group 5 is slightly improved.

in the HE staining of DIO liver sections. The finding of fatty livers in these mice also explained the increase in serum ASL and ALT levels. As expected, steatosis was obviously improved in the treatment groups, in line with the decrease in serum lipids and transaminase levels (Figures 5 and 6).

3.2.6. Activation of p-AMPK and LC3. To investigate the relationship between the improvements in metabolic conditions and AMPK activation, we examined the levels of AMPK phosphorylation in liver, skeletal muscle, and cardiac muscle. The results showed that there was no significant difference in AMPK phosphorylation among groups in any of these tissues (Figure 7), implying the AMPK activation does not contribute to the long-term benefits of caloric restriction. Next, we evaluated the autophagy activity in cardiac muscle as represented by LC3 II/LC3 I ratio. The results demonstrated that autophagy activity was significantly elevated in Groups 4 and 6, and such trend was also quite obvious in Group 3 as compared with Group 2 (Figure 7). Considering the facts that these three groups were treated with caloric restriction and showed consistent improvement in metabolic indices, it strongly indicates autophagy activity is responsible for the metabolic improvements in caloric restriction conditions.

4. Discussion

The activation of AMPK has been considered as an important factor in adapting the body to the state of energy stress. Upon sensing an increased level of AMP/ATP ratio, AMPK

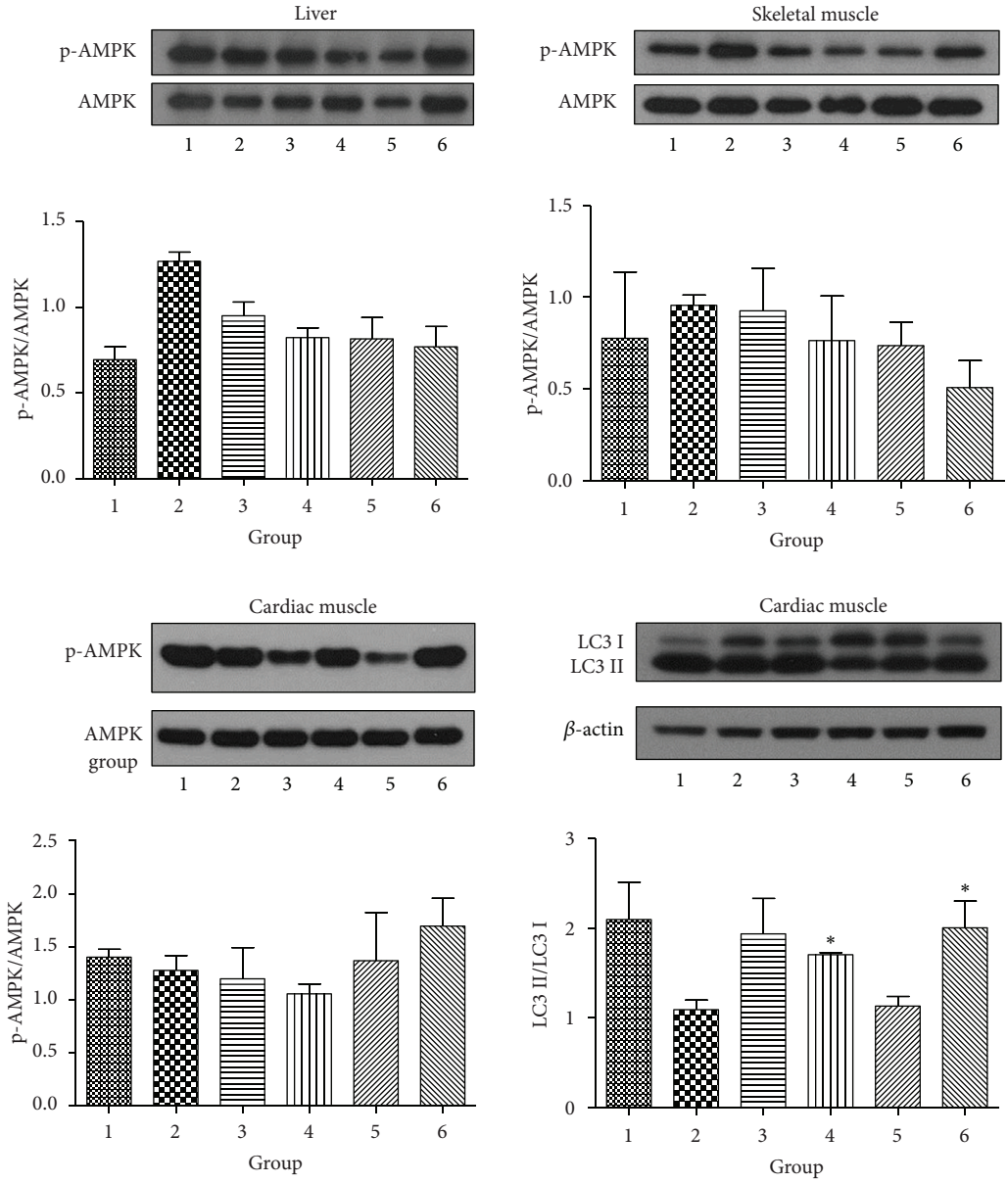

Figure 7: Representative images of the immunoblotting of AMPK in liver, skeletal muscle, cardiac muscle, and LC3 in cardiac muscle. The levels of LC3 II exhibit a significant increase in Groups 4 and 6 compared with that in Group 2, and such trend can also be seen in Group 3. On the other hand, no significant difference in p-AMPK is found in liver, skeletal muscle, or cardiac muscle.

can be fully activated by an increased phosphorylation level at Thr^{172}. Activated AMPK can interact with numerous pathways including that of SIRT, mTOR, and autophagy and regulate the activities including mitochondrial biogenesis, glucose uptake, and lipid metabolism [4]. The functions of AMPK imply that its activation may participate in mediating the beneficial effects of chronic caloric restriction and exercise. However, whether AMPK activation plays a part in caloric restriction and exercise is still controversial [5]. In this study, high fat diet was used to induce the obese mice models, and then interventions of CR and exercise were used to improve the metabolic conditions. After that, we examined the level of AMPK phosphorylation in liver, skeletal muscle, and heart tissues to investigate the relationship between metabolic improvement and AMPK activation.

As expected, two-month feeding of high fat diet successfully induced the obesity and altered the metabolic state of the model mice, which was characterized by increased fat accumulation, insulin resistance, and impaired liver function. However, to most of the indices, exercise alone failed to make a significant improvement, while the effects of caloric restriction were quite prominent. Though it is plausible that exercise alone did not have an obvious effect, which may be caused by an insufficient dosage, it did have a synergistic effect when combined with CR. Moreover, because we are more concerned with the correlation between metabolic

improvement and levels of signaling proteins, instead of the effects of individual interventions, this result should not preclude us from getting a reasonable conclusion. Our results indicate that the activation of AMPK is not closely correlated with the improvement of the metabolic conditions of the diet induced obese mice. Thus, the long-term beneficial effects of chronic CR and exercise may not be mediated through AMPK activation.

The result on AMPK in our study is in consistence with that in the study of Gonzalez et al. [13], which showed AMPK activation did not respond to chronic caloric restriction or even fasting in skeletal muscle, cardiac muscle, or liver. However, these results appeared to be contradictory to some other studies, in which CR was shown to lead to AMPK activation [14–16]. In the study of Edwards et al. [14], it was shown that a life-long CR was able to protect the myocardium against ischemia/reperfusion (I/R), and this protective effect was demonstrated to involve AMPK activation, because inhibition of AMPK activation was able to abolish the protection.

Indeed, AMPK can interact with multiple pathways that may assist in the adjustment of body to different energy metabolic states, and there is also pharmacologic evidence supporting the role of AMPK in mediating CR effects, in which resveratrol was shown to activate AMPK by decreasing ATP production [17]. Therefore, the question why there are different observations concerning AMPK activation in chronic CR and exercise still needs to be clarified. In a previous study, it was proposed that a lack of an effect of CR on AMPK activity is probably due to that the energetic challenge presented by CR was not severe enough to cause the changes in ATP and AMP to activate AMPK. This level of energy challenge brought by CR, however, should be sufficient to affect the metabolic states of mice as shown in both their and this study [13]. Therefore, this may be an explanation on why chronic CR and exercise did not induce the activation of AMPK, and further, it suggests that the beneficial effects of chronic CR and exercise are mediated by some other pathways instead of AMPK activation.

It is possible that AMPK only becomes activated in more severe and acute energy deprival conditions. For instance, in the study of Edwards et al. [14], a life-long CR could indeed protect myocardium from I/R injuries accompanied by an increase in p-AMPK level, but this does not indicate that AMPK became activated in the basal condition. Also, the cancelling of protection by AraA only proves the AMPK was activated in I/R, which represented an acute and a much more severe energy stress situation than that in chronic CR or exercise. However, the more active AMPK during I/R after a life-long CR does imply a mechanism by which AMPK was sensitized by long-term CR.

If these effects are not mediated by AMPK, then there must be other mechanisms that explain why chronic CR and exercise may improve the metabolic conditions. Recent findings have suggested a relationship between autophagic activity and metabolic conditions. The study of Yang et al. [7] showed that loss of autophagy might be responsible for the insulin resistance in obesity, which was characterized by the downregulation of Atg7, and restoration of Atg7 was able to improve the glucose tolerance in mice. In another research, both long-term and short-term exercises were shown to be able to improve the insulin sensitivity, and this effect depended on induced autophagy [8]. In the light of these findings, we examined the level of conversion of unlipidated LC3 I to LC3 II (autophagosome-membrane-associated lapidated form) in the heart tissues. The results clearly exhibited a trend which is consistent with the change in metabolic conditions in the interventional groups. Specifically, a significant increase in autophagy activity was found in Group 6 mice, and this group also showed a much more prominent trend in increased insulin level, which indicated an improvement in insulin sensitivity. Also, significant increase in the antidiabetic adiponectin, decrease in serum lipids, and improvement in liver function were also exhibited. Therefore, this may be biochemical evidence that an enhanced autophagy activity may participate in regulating the metabolic conditions during chronic CR and exercise, and the effect of autophagy may further extend beyond glucose homeostasis. Moreover, although autophagy in living cells is under control of AMPK, the induction in this context possibly does not depend on AMPK activation.

Since autophagy in living cells is known to be under the regulation of multiple pathways including AMPK, SIRT, and mTOR, it may be important to investigate which among these pathways is responsible for the activation of autophagy during chronic CR and exercise. In the study of He et al., the activation of autophagy in short-term exercise was shown to involve Bcl2-beclin1 complex. Upon dissociating with the antiautophagic Bcl2, beclin1 can activate autophagy, which is mediated via phosphorylating AMPK [8]. However, the long-term exercise might act through a different pathway according to our data, since the p-AMPK level did not change significantly in the groups with long-term exercise. The further study on SIRT and mTOR pathway activity might be helpful to find the molecules responsible for autophagy activation during chronic CR and exercise.

5. Conclusions

Our results showed that AMPK activation, which was represented by the level of p-AMPK, did not correlate with the improvement of metabolic conditions in DIO mice, implying AMPK activation may not participate in mediating the beneficial effects of chronic CR or exercise. On the other hand, however, we found the autophagy activity might be related to the improved metabolic conditions but was not correlated with AMPK activation. Thus, we propose that autophagy may play a role in mediating the effects of chronic CR and exercise, but it is not regulated by AMPK activity. However, this hypothesis based on the findings in our research still requires confirmation. For example, AMPK knockout mice might be used to check if these interventions are still effective in the absence of AMPK activation. Finally, detecting SIRT and mTOR pathway activity in the samples may also provide clues on the regulation of autophagy activity in obese mice that received chronic CR or exercise treatment.

Conflict of Interests

The authors declare they have no conflict of interests.

Acknowledgments

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