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ORIGINAL PAPER

3T3-L1 adipocytes induce dysfunction of MIN6 insulin-secreting cells via multiple pathways mediated by secretory factors in a co-culture system

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Abstract Pancreatic β-cell dysfunction is an important pathological change in type 2 diabetes, which is tightly related to obesity. However, the direct role of adipose tissue in β-cell dysfunction has not been well understood. In this study, we examined the effects of 3T3-L1 adipocytes on MIN6 insulin-secreting cells in a co-culture system. MIN6 cells used here kept most of β-cell functions but less sensitive to glucose stimulation. Tolbutamide, the K_{ATP} channel blocker, was therefore used to stimulate insulin secretion in this report. MIN6 cells co-cultured with 3T3-L1 adipocytes had significantly reduced intracellular calcium concentration ($[Ca^{2+}]_i$) and lost the ability to secrete insulin in response to tolbutamide, compared to the control cells. 3T3-L1 adipocytes significantly decreased the expression of *insulin, glucokinase* and *Kir6.2* genes but increased the expression of uncoupling protein-2 (UCP-2) in MIN6 cells after one week of co-culture, as measured by semi-quantitative RT-PCR. 3T3-L1 adipocyte-conditioned medium also significantly decreased insulin secretion and the expression of *insulin, glucokinase* and *Kir6.2* genes in MIN6 cells. The conditioned medium also reduced tyrosine kinase activity in MIN6 cells. The inhibitor of protein tyrosine kinase, genistein, decreased the expression of *glucokinase* and *Kir6.2* in MIN6 cells, while two free fatty acids, oleic acid and linoleic acids, were found to increase *UCP-2* expression. The present study demonstrates that 3T3-L1 adipocytes directly impair insulin secretion and the expression of important genes in MIN6 cells. The effects of 3T3-L1 adipocytes on MIN6 cells are ascribed to secreted bioactive factors and may be mediated via multiple pathways, which include the upregulation of *UCP-2* expression via free fatty acids, and downregulation of *glucokinase* and *Kir6.2* expression via decreasing protein tyrosine kinase activity.

Keywords Adipocyte · Insulin · Glucokinase · Kir6.2 · UCP-2 · Co-culture

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Introduction

Pancreatic β-cell dysfunction plays a key role in the pathogenesis of type 2 diabetes [1]. β-cells in normal function secrete insulin under the stimulation of glucose to maintain the normal blood glucose level. Glucose stimulates insulin secretion by generating adenosine triphosphate (ATP) in mitochondria of β-cells, which subsequently induces a closure of ATP-sensitive potassium channels (K_{ATP} channels), an activation of voltage-gated calcium channels ($Ca^{2+}_{(V)}$ channels) and Ca^{2+} influx. The resulted increase in intracellular calcium ($[Ca^{2+}]_i$) stimulates exocytosis of insulin granules. β-cell dysfunction is characterized by defective glucose-stimulated insulin secretion (GSIS) [2], and concomitant with marked changes in expression of several important genes at mRNA and protein levels as demonstrated in the study in islets from Zucker Diabetic Fatty (ZDF) rats [3]. Key genes involved in insulin secretion such as *insulin, glucokinase* (*GK*), *potassium channels,* Ca^{2+}*-ATPase and* $Ca^{2+}_{(V)}$ *channels* were down-regulated in islets of ZDF rats [3]. This can explain the defect in GSIS in β-cell dysfunction, but not the mechanism of pathogenesis of β-cell dysfunction, which is still not fully understood.

Once diabetes is established, chronic hyperglycemia and hyperlipidemia exert deleterious effects on β-cell function,

referred to as glucotoxicity and lipotoxicity [4–6]. However, these factors can not fully explain all characteristics and pathogenesis of β-cell dysfunction. For example, studies in ZDF rats show that β-cell dysfunction has taken place before the development of hyperglycemia [3], and lipotoxicity is dependent on the existence of hyperglycemia [7, 8]. Therefore, other unidentified factors must be involved. Enlargement of adipose tissue (obesity) and presence of adipocyte-derived factors may provide an explanation as to the pathogenesis of β-cell dysfunction.

Obesity is a high risk factor for type 2 diabetes, and it is well known that obesity leads to type 2 diabetes by inducing insulin resistance [9]. It is unknown, however, whether adipose tissue plays direct role in inducing β-cell dysfunction. Because most patients with type 2 diabetes are obese for a long time before hyperglycemia and dyslipidemia arise [10], it is possible that adipose tissue leads to β-cell dysfunction ahead of high levels of circulating glucose and free fatty acids. Recently, it has been known that adipose tissue has endocrine function. It secretes leptin, resistin, adiponectin and tumor necrosis factor-α (TNF-α) and releases free fatty acids (FFAs) [11]. The receptors for some of these factors have been found on pancreatic β-cells [12–14] and the direct actions of some adipocyte-secreted factors on pancreatic β-cells have been reported. For example, leptin and TNF-α have inhibitory effects on insulin secretion, and FFAs have a lipotoxic action on β-cells. However, the effects of adipocytes on β-cells are unclear due to the increasing number of recently discovered factors. Furthermore, little is known about the combined effects of the adipocyte-secreted factors on pancreatic β-cells. In this study, we investigate the integrated effects of 3T3-L1 adipocytes on MIN6 insulin-secreting cells using a co-culture system by measuring gene expression and insulin secretion. The results support the direct involvement of adipocytes in β-cell dysfunction via multiple pathways mediated by secretory factors.

Results

Expression of adipocyte-secreted factors in 3T3-L1 adipocytes

We firstly observed gene expression in 3T3-L1 cells using RT-PCR. Differentiated 3T3-L1 adipocytes and fat tissues from mouse epididymis fatty pad expressed adipocyte-specific factors such as leptin, adiponectin and resistin at similar levels. TNF-α was expressed weakly in fat tissues and was undetectable in differentiated 3T3-L1 adipocytes. Undifferentiated 3T3-L1 pre-adipocytes showed non-detectable expression of these factors. A representative result of five separate experiments was shown in Fig. 1. These results demonstrate that the differentiated 3T3-L1 adipocytes display similar genetic characteristics to in vivo adipocytes and can be used to observe the effects of adipocytes on insulin-secreting cells.

Effects of 3T3-L1 adipocytes on insulin secretion from MIN6 cells

3T3-L1 adipocytes significantly inhibited insulin secretion from MIN6 cells. MIN6 cells had a basal level of insulin secretion in 2 mM glucose at a rate of 10.64 ± 1.90 μU/min/μg RNA in the control group and 6.66 ± 1.05 μU/min/μg RNA in the co-culture group ($P < 0.01$). As MIN6 cells used in this experiment did not respond normally to high glucose, K_{ATP} channel blocker, tolbutamide, was used to check stimulated insulin secretion. Insulin secretion from control cells was significantly increased by 0.1 mM tolbutamide (75.72 ± 4.77 μU/min/μg RNA, $P < 0.01$ vs. basal level), whereas MIN6 cells co-cultured with 3T3-L1 adipocytes showed no response to tolbutamide in insulin secretion (8.67 ± 1.58 μU/min/μg RNA; $P > 0.1$ vs. control group; Fig. 2). Cell numbers were counted with no difference between control and co-culture groups.

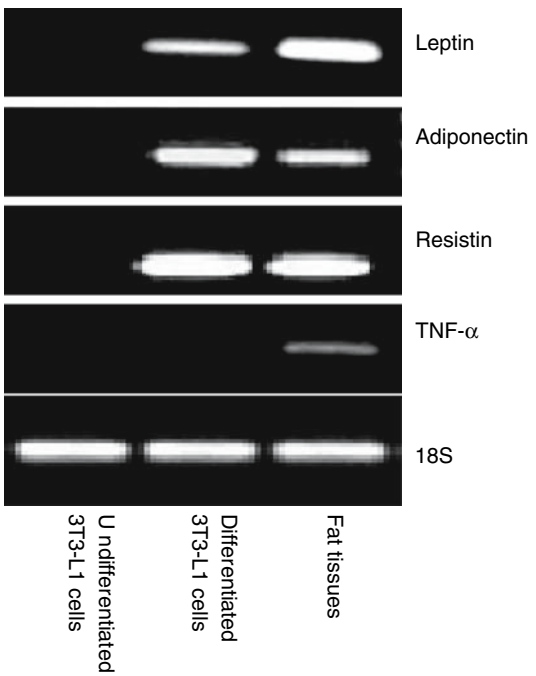

Fig. 1 The expression of adipocyte-secreted factors in 3T3-L1 cells. Leptin, resistin and adiponectin are expressed in differentiated 3T3-L1 adipocytes and in fat tissues from mouse epididymis fat pad, but not in undifferentiated 3T3-L1 preadipocytes. TNF-α expression was weakly detected in fat tissues

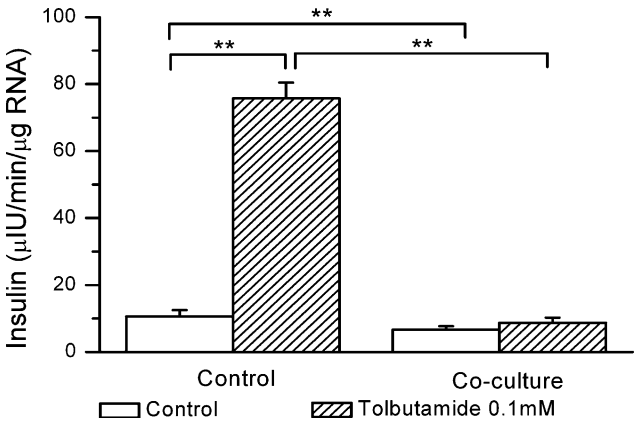

Fig. 2 The effects of 3T3-L1 adipocytes on insulin secretion from MIN6 cells. The base level of insulin secretion at 2 mM glucose was significantly decreased in MIN6 cells co-cultured with 3T3-L1 adipocytes as compared with the control group. Tolbutamide (0.1 mM) stimulated significant increase in insulin secretion in the control group, but not in the co-culture group. The column represents the mean ± SEM of three separate experiments. $^{**}P < 0.01$ between groups

Effects of 3T3-L1 adipocytes on $[Ca^{2+}]_i$ in MIN6 cells

$[Ca^{2+}]_i$ measurement showed that co-culture with 3T3-L1 adipocytes impaired the increase in $[Ca^{2+}]_i$ in MIN6 cells. The resting levels of $[Ca^{2+}]_i$ were not significantly different between MIN6 cells under control conditions and MIN6 cells co-cultured with 3T3-L1 adipocytes (0.204 ± 0.035 in control group vs. 0.214 ± 0.021 in co-culture group). $[Ca^{2+}]_i$ in control cells increased significantly after stimulation by 0.1 mM tolbutamide (Fig. 3A), but this was significantly attenuated in cells co-cultured with 3T3-L1 adipocytes (0.306 ± 0.026 in control vs. 0.238 ± 0.016 in co-culture group) (Fig. 3B, C).

Effects of 3T3-L1 adipocytes on gene expression in MIN6 cells

Gene expression in MIN6 cells was also changed by 3T3-L1 adipocytes. *Insulin, GK, Kir6.2* (the K_{ATP} channel subunit), and uncoupling protein-2 (*UCP-2*) are important in regulating insulin secretion. Their expression in MIN6 cells was not changed by 1 week of co-culture with 3T3-L1 pre-adipocytes, but was significantly modulated by 3T3-L1 adipocytes. In MIN6 cells co-cultured with 3T3-L1 adipocytes, the expression *of insulin, GK and Kir6.2* mRNA was significantly decreased to 87% (0.872 ± 0.026), 57% (0.566 ± 0.029) and 69% (0.692 ± 0.028), respectively, of that in control cells. UCP-2 expression was significantly increased to 116% (1.159 ± 0.015) of that of the control (Fig. 4B). The electrophoresis images of RT-PCR products showed that the cDNA bands were in accordance with the designed sizes of amplified gene sequences, and subsequent sequencing also confirmed the products of target genes (Fig. 4A).

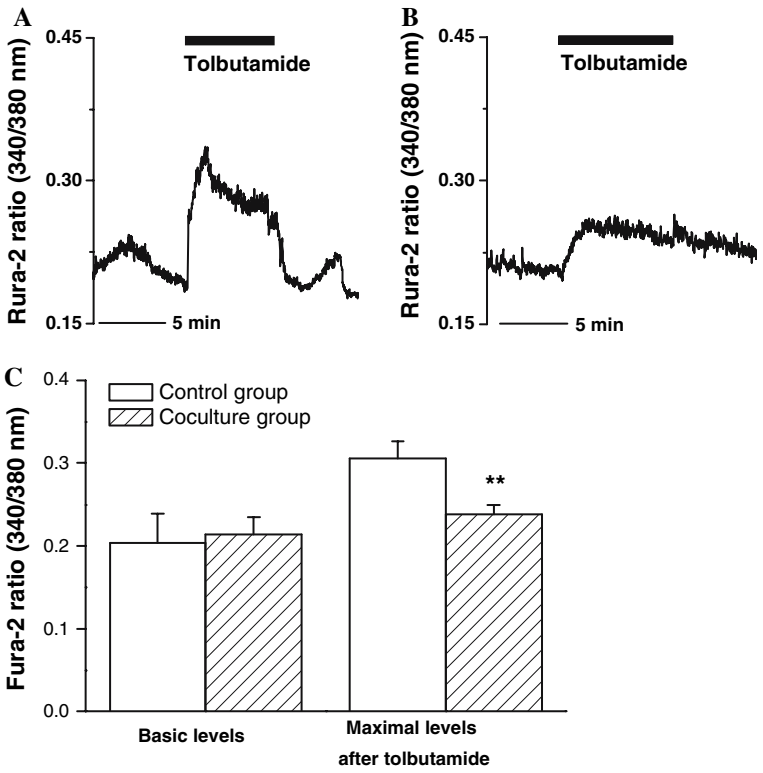

Fig. 3 The effects of 3T3-L1 adipocytes on the intracellular calcium concentration in MIN6 cells. Control MIN6 cells responded to tolbutamide (0.1 mM) with a dramatic increase in $[Ca^{2+}]_i$ as shown in panel (**A**). In contrast, MIN6 cells co-cultured with 3T3-L1 adipocytes for one week had attenuated tolbutamide-stimulated increase in $[Ca^{2+}]_i$ as shown in panel (**B**). The statistical analysis is shown in panel (**C**). The column represents the mean ± SEM of six separate experiments. $^{**}P < 0.01$ (vs. the maximal levels in control group)

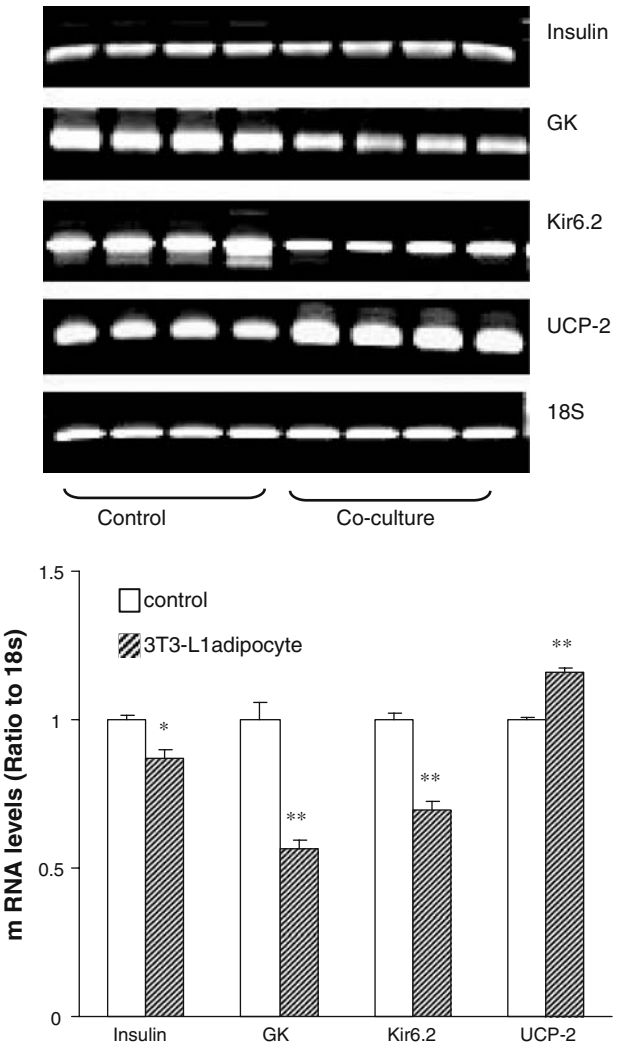

Fig. 4 The effects of 3T3-L1 adipocytes on gene expression in MIN6 cells. The bands in the upper panel were the result of electrophoresis, in which the left is the control group and the right is the co-culture group. As shown in lower panel, expression of *insulin*, *GK* and *Kir6.2* was significantly decreased and *UCP-2* expression was significantly increased in MIN6 cells after one week co-culture with 3T3-L1 adipocytes as compared with the control. The column represents the mean ± SEM of four separate experiments. $*P < 0.05$; $**P < 0.01$ (vs. control)

Effects of 3T3-L1 adipocyte-conditioned medium on gene expression and insulin secretion of MIN6 cells

We observed the effects of 3T3-L1 adipocyte-conditioned medium on gene expression and insulin secretion of MIN6 cells to confirm that the effects of 3T3-L1 adipocytes on MIN6 cells were achieved via secreted factors in the culture medium. In fact, in co-culture system, there was no physical contact between the two cell types. Gene expressions of *insulin, GK* and *Kir6.2* was significantly decreased to 0.903 ± 0.014, 0.705 ± 0.024 and 0.676 ± 0.041 respectively of control levels in MIN6 cells treated with the conditioned medium. It is accordant to the changes of gene expression in MIN6 cells co-cultured for 1 week with 3T3-L1 adipocytes. However, *UCP-2* expression was not increased by the conditioned medium.

Insulin secretion in 48 h of cell culture in conditioned medium was reduced in MIN6 cells cultured with 3T3-L1 adipocyte-conditioned medium, but not in 3T3-L1 preadipocyte-conditioned medium (Fig. 5A). Interestingly, insulin content was similar between all groups (Fig. 5B) indicating that storage of insulin is sufficient, but secretion of insulin is inhibited, under the adipocyte-conditioned medium culture condition.

Effects of free fatty acids on gene expression in MIN6 cells

Free fatty acids (FFAs) are important adipocyte-derived factors. In this study, oleic acid and linoleic acid were used

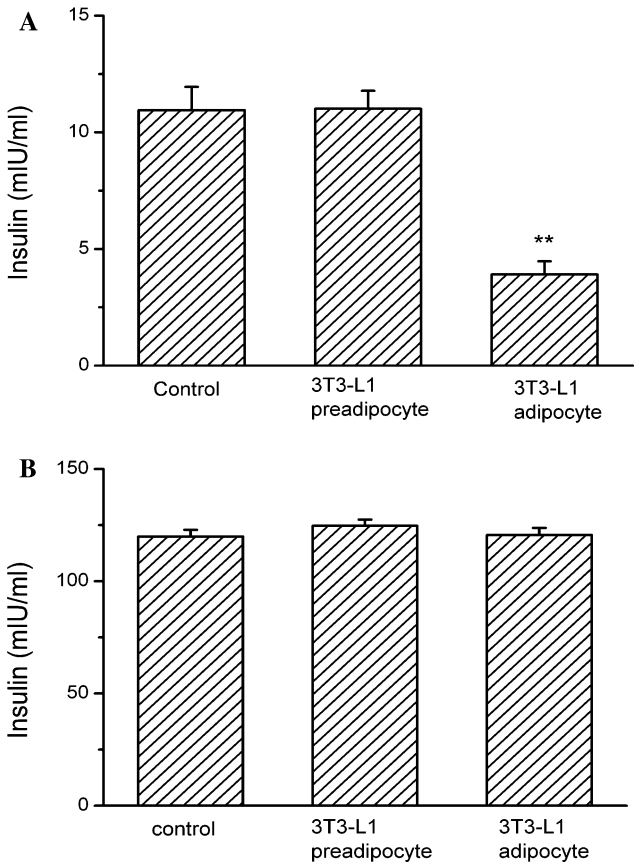

Fig. 5 The effects of 3T3-L1 adipocyte-conditioned medium on insulin secretion from MIN6 cells. Insulin levels in culture medium of MIN6 cells were significantly decrease by treatment with 3T3-L1 adipocyte-conditioned medium, but not by treatment with 3T3-L1 preadipocyte-conditioned medium (**A**). Insulin contents in MIN6 cell lysis were not significantly different among three groups (**B**). The column represents the mean ± SEM of 4 separate experiments. $**P < 0.01$ (vs. control and preadipocyte-conditioned medium culture groups)

to confirm whether FFAs are responsible for the changes in gene expression in MIN6 cells co-cultured with 3T3-L1 adipocytes. Oleic acid increased UCP-2 expression by 1.221 ± 0.042 and linoleic acid by 1.381 ± 0.051 of control levels. Unexpectedly, both oleic acid (0.1 mM) and linoleic acid (0.1 mM) significantly increased Kir6.2 expression by 1.213 ± 0.045 and 1.198 ± 0.016, respectively, after 2 days of treatment. *Insulin* and *GK* expression in MIN6 cells were not changed by either oleic or linoleic acid (Fig. 6).

Effect of 3T3-L1 adipocyte-conditioned medium on tyrosine kinase activity in MIN6 cells

Glucokinase expression is mainly regulated via insulin signaling, and a decrease in insulin action on β-cells may be the cause of inhibited expression of *GK*. A major signal of the insulin receptor is the activation of tyrosine kinases, so we measured total protein tyrosine kinase activity in MIN6 cells cultured with normal culture medium, 3T3-L1 preadipocyte-conditioned medium and 3T3-L1 adipocyte-conditioned medium for 1 week. The relative tyrosine kinase activities on IRS-1 and IR subunit normalized by protein levels for normal culture medium, 3T3-L1 preadipocyte-conditioned medium or 3T3-L1 adipocyte-conditioned medium were shown in Fig. 7. Results indicate that both 3T3-L1 preadipocyte-conditioned medium and 3T3-L1 adipocyte-conditioned medium significantly reduced tyrosine kinase activities, with significantly more inhibition occurring with 3T3-L1 adipocyte-conditioned medium.

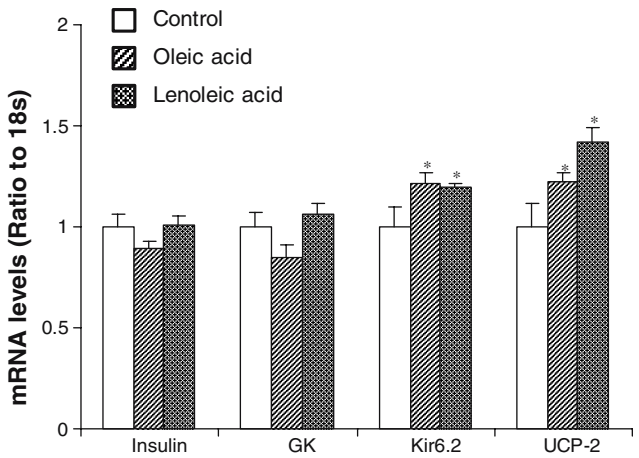

Fig. 6 The effects of free fatty acids on gene expression in MIN6 cells. Oleic acid and linoleic acid increased the expression of *UCP-2* and *Kir6.2* in MIN6 cells. The column represents the mean ± SEM of four separate experiments. *$P < 0.05$ (vs. control group)

Effects of inhibition of tyrosine kinase activity on gene expression in MIN6 cells

We next observed the effects of inhibiting tyrosine kinase activity on gene expression. Genistein is a selective inhibitor of protein tyrosine kinases and was used here to investigate the effect of tyrosine kinase inhibition in MIN6 cells. Genistein (0.1 mM, 24 h treatment) significantly decreased *GK* and *Kir6.2* expression to 0.706 ± 0.016 and 0.671 ± 0.044 of control levels respectively. *Insulin* and *UCP-2* expression were not changed by genistein (Fig. 8). Genistin, a structurally similar compound without the inhibitory effect on tyrosine kinases, was used as a negative control and no change was found on expression of any target molecules. Treatment of cells with estradiol up to 100 nM for 24 h did not change the expression level of *GK* and *Kir6.2* (data not shown). This excludes the possibility that the estradiol-like effects of genistein contribute to the regulation of GK and Kir6.2 expression.

Discussion

The present study showed that factors derived from 3T3-L1 adipocytes directly impair normal function of MIN6 insulin-secreting cells by attenuating insulin secretion, inhibiting the increase in $[Ca^{2+}]_i$ and changing gene expression. Results suggest that adipocytes have integrated deteriorating effects on β-cell function which supply a new insight into the mechanism of β-cell dysfunction in type 2 diabetes.

3T3-L1 adipocytes induced dysfunction of MIN6 insulin-secreting cells by inhibiting the expression of *insulin, glucokinase* and *Kir6.2*. We have reported the regulation of Kir6.2 expression by 3T3-L1 adipocytes [15]. Kir6.2 is the α subunit of the K_{ATP} channel and forms the ion pore of the channel [16]. The K_{ATP} channel is the pivotal link between glucose metabolism and the electrical activity of pancreatic β-cells, and is important in regulating glucose- and sulfonylurea-stimulated insulin secretion. A decrease in *Kir6.2* expression leads to the loss of glucose- and sulfonylurea-stimulated insulin secretion [17, 18]. A decrease in insulin expression results in a low level of insulin synthesis and storage and subsequently impairs insulin secretion. Glucokinase is the enzyme catalyzing glucose phosphorylation, and it determines the rate of glucose metabolism in pancreatic β-cells [19, 20]. Decreased glucokinase expression in MIN6 cells would lead to the inhibition of glycolysis and impair GSIS. It has been supported by the observation that defects in glucokinase gene expression contribute to the genetic susceptibility to type 2 diabetes [21, 22]. The decrease in gene expression of *insulin, GK* and *Kir6.2* results in a defect of GSIS. The defect of insulin secretion was confirmed in the MIN6 cells co-cultured with

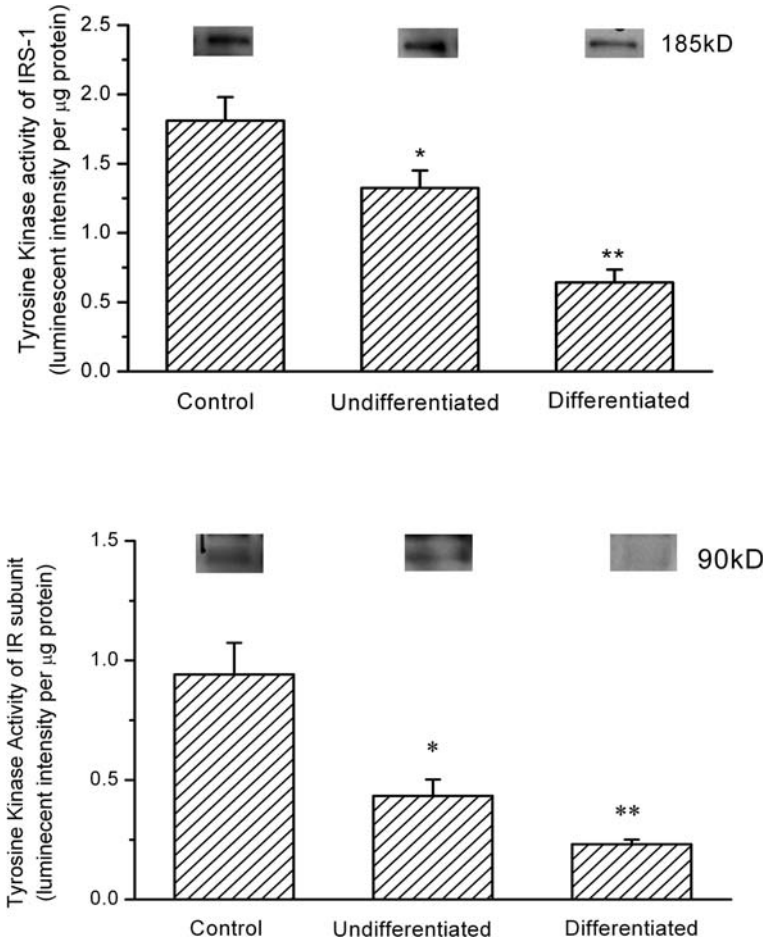

Fig. 7 The effects of 3T3-L1 adipocyte-conditioned medium on protein tyrosine phosphorylation of IR β-subunit and IRS-1 in cultured MIN6 cells. In cell lysis preparation, tyrosine phosphorylation of IRS-1 (**A**) and IR β-subunit (**B**) was significantly reduced in MIN6 cells treated with 3T3-L1 adipocyte-conditioned medium and 3T3-L1 preadipocyte-conditioned medium. 3T3-L1 adipocyte-conditioned medium evoked more significant reduction compared with 3T3-L1 preadipocyte-conditioned medium. The column represents the mean ± SEM of 4 separate experiments. *$P < 0.05$; **$P < 0.01$ (vs. control medium culture group)

3T3-L1 adipocytes. Because MIN6 cells we used in this study were not sensitive to glucose in insulin secretion, we used tolbutamide to stimulate insulin secretion because

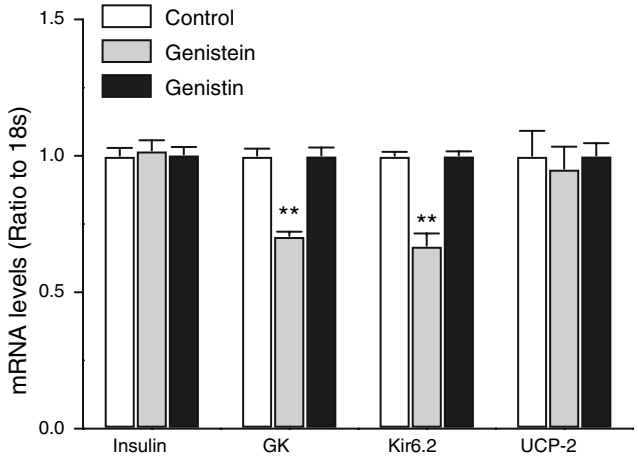

Fig. 8 The effects of genistein on gene expression in MIN6 cells. Genistein (0.1 mM), but not genistin, treatment for one day significantly decreased the expression of GK and Kir6.2 in MIN6 cells as compared with the control group. The column represents the mean ± SEM of four separate experiments. **$P < 0.01$ (vs. control and genistein groups)

tolbutamide may imitate the electrical response of β-cells to glucose in insulin secretion. The results of insulin secretion combined with that of gene expression strongly indicate that 3T3-L1 adipocytes induced dysfunction of MIN6 cells. On the other hand, 3T3-L1 adipocytes increased the expression of UCP-2 mRNA. UCP-2 uncouples the oxidation of glucose from phosphorylation and results in the attenuation of ATP generation. Reports have concluded that an increase in *UCP-2* expression leads to the inhibition of GSIS [23–25]. Therefore, although up-regulation of UCP-2 is not the reason for the inhibition of tolbutamide-stimulated insulin secretion in this study, the increase in UCP-2 expression may contribute to the dysfunction of β-cells induced by adipocytes.

$[Ca^{2+}]_i$ level is important in regulating insulin secretion. Upon the closure of K_{ATP} channels by tolbutamide, control MIN6 cells demonstrated a quick elevation in $[Ca^{2+}]_i$, a response which was attenuated in co-cultured MIN6 cells. Inhibition of protein tyrosine kinases in mouse pancreatic islet cells reduces the expression of voltage-dependent L-type calcium channels [26]. The activity of protein tyrosine kinases in MIN6 cells co-cultured with 3T3-L1 adipocytes is significantly decreased, which reduces the

expression and synthesis of calcium channels in MIN6 cells. This may be the reason for the inhibition of $[Ca^{2+}]_i$ response to tolbutamide. The weak response of $[Ca^{2+}]_i$ to the stimulants also leads to reduced insulin secretion.

3T3-L1 adipocytes-induced dysfunction of MIN6 cells is mediated by its secretory factors. No physical contact exists between the adipocytes and MIN6 cells in the co-culture system, so any interaction between them occurs through factors secreted into the medium. This view is strongly supported by the results that 3T3-L1 adipocyte-conditioned medium decreased gene expression of *insulin, GK* and *Kir6.2* to the same levels as 3T3-adipocytes did. To investigate the mechanism of 3T3-L1 adipocyte-induced dysfunction of MIN6 cells, we observed the effects of FFAs on gene expression in MIN6 cells, as FFAs released from adipocytes are well known to influence β-cell function [27–29]. It has been previously demonstrated that FFAs such as oleic acid increase UCP-2 expression and impair the normal functioning of insulin-secreting cells [30–32]. Oleic and linoleic acids, which are abundant in adipocytes, increased the expression of *UCP-2* in MIN6 cells. It is inferred that FFAs released from 3T3-L1 adipocytes are responsible for the up-regulation of UCP-2 expression in MIN6 cells. Oleic and linoleic acids also increased *Kir6.2* expression in MIN6 cells. Although the intracellular mechanism of this effect remains unknown, it may involve a specific FFA receptor, GPR40, which was recently discovered [33, 34].

Other secretory factors may also take part in the action of 3T3-L1 adipocytes on MIN6 cells. *GK* expression, regulated by insulin signaling, is down-regulated by 3T3-L1 adipocytes. The insulin receptor is a tyrosine kinase receptor which elicits the tyrosine kinase activity of many downstream signaling molecules after activation by insulin. We studied the tyrosine kinase activity in MIN6 cells treated with 3T3-L1 adipocyte-conditioned medium and found that conditioned medium inhibited kinase activity. Furthermore, we observed the effects of an inhibitor of tyrosine kinases, genistein, on MIN6 cells. Genistein is an inhibitor of tyrosine-specific protein kinases and is used widely as a protein tyrosine kinase inhibitor in many researches [35, 36]. Our results show that genistein decreased the expression of *GK* and *Kir6.2*. It is therefore plausible that 3T3-L1 adipocyte-derived factors decrease the expression of *GK* and *Kir6.2* by decreasing protein tyrosine kinase activities or insulin resistance.

3T3-L1 cells, the widely used cell model of mouse adipocytes, maintain the genetic characteristic of in vivo adipocytes, as they express adipocyte-secreted factors after differentiation. MIN6 cells are derived from mouse pancreatic β-cells and are known to maintain the basic function of β-cells [37, 38]. If the adipocytes have similar effects on pancreatic β-cells in vivo, it could be concluded that adipocytes play an important, albeit not fully clarified, role in the etiology of β-cell dysfunction in type 2 diabetes. Identification in future studies of the specific adipocyte-secreted factors that are involved in the dysfunction of β-cells will shed light on the etiology of pancreatic β-cell function in type 2 diabetes.

Materials and methods

Culture of 3T3-L1 cells and MIN6 insulin-secreting cells

3T3-L1 cells (American Type Culture Collection) were plated onto plastic dishes (100 mm in diameter) and differentiation was induced by insulin. MIN6 cells were cultured in plastic dishes (35 mm in diameter) without edges. The plastic dishes with MIN6 cells were then put into the dishes containing undifferentiated 3T3-L1 pre-adipocytes or into the dishes containing differentiated 3T3-L1 adipocytes for co-culture. After 1 week of co-culture, MIN6 cells were harvested for RNA extraction or used to measure insulin secretion. For intracellular calcium measurement, MIN6 cells were cultured on glass cover slips coated with 0.01% Poly-L-Lysine and put into the plastic dishes with 3T3-L1 adipocyte for co-culture. 3T3-L1 cells and MIN6 cells were co-cultured in complete medium (DMEM medium containing 25 mM glucose plus 10% FCS (v/v), 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μM 2-mercaptoethanol). The volume of medium for co-culture system was 400 μl/cm^2. The medium were refreshed every day. MIN6 cells in the control group were cultured in plastic dishes (100 mm in diameter) without plantation of 3T3-L1 cells.

To observe the effects of medium conditioned with 3T3-L1 adipocytes on MIN6 cells, the conditioned medium was gathered and mixed with the fresh complete medium in the proportion of 1:1 and filtered through a 0.22 μm filter. Medium from MIN6 cell culture after two days in conditioned medium was collected for insulin assay. MIN6 cells were harvested for RNA extraction after culture for 1 week.

To observe effects of FFAs and genistein on gene expression of MIN6 cells, cells were cultured in 6-well plates. At 80% confluence, the medium was refreshed and MIN6 cells in different groups were treated with 0.1 mM oleic acid for 2 days, 0.1 mM linoleic acid for 2 days or 0.1 mM genistein for 1 day. After treatment, the cells were harvested for RNA extraction.

Semi-quantitative RT-PCR

The total RNA from MIN6 cells and 3T3-L1 cells was extracted using a previously reported method [39]. Reverse

transcription was performed in the reaction system containing 1 μg RNA, reverse transcriptase, DNA polymerase and random primer, and the cDNA obtained was used to do PCR with each pair of primers. Primers were designed in this laboratory or cited from the published paper [40]. The sequences of the primers are given in Table 1. The conditions, including annealing temperature and appropriate cycle number for each pair of primers, were determined in preliminary experiments. The general procedure of PCR was as follows: after denaturing for 5 min at 94°C, samples were cycled through denaturing at 94°C for 30 s, annealing for 30 s at annealing temperature and extending at 72°C for 30 s. In the last cycle, the extending time was increased to 10 min. The PCR products were run by electrophoresis in 1.4% agarose gel, then stained by ethidium bromide and photographed through the UV system. The photos were transferred to a computer and the intensity of the bands was quantified using the software Quantity One (Life Science). The mRNA levels of the genes amplified were normalized to those of 18S for each sample.

Intracellular calcium measurement

MIN6 cells were co-cultured on coverslips with 3T3-L1 adipocytes for 1 week, and then loaded with 2 μM Fura-2/AM for 40 min at 37°C. The cover slips with loading cells were used as exchangeable bottoms of a chamber containing 500 μL medium. The chamber was placed on the stage of an inverted microscope (Olympus) equipped with a 40× UV fluorite objective. These cells were illuminated alternatively at 340 nm and 380 nm, and emission was measured at 510 nm with a photomultiplier. Fluorescence signals at the two wavelengths of excitation light were transferred into the computer by Digidata 1200 and were recorded using Axon software AxoScope 8.0. The ratio of fluorescence signals 340/380 nm indicated levels of $[Ca^{2+}]_i$. The solution for $[Ca^{2+}]_i$ measurement was composed of (mM): NaCl 140, KCl 4.7, $CaCl_2$ 2.6, $MgCl_2$ 1.2, $NaHCO_3$ 1, KH_2PO_4 1.2, Glucose 2 and HEPES 5 (pH = 7.4 with 1 M NaOH).

Insulin assay

MIN6 cells were washed with serum-free solution (as above) and treated with different stimulants in the solution at 37°C for 20 min. The culture medium and incubation solution were collected to measure insulin levels using RIA kit (Linco Research, America) or ELISA kit (DSL Laboratory, Sydney, Australia). Control groups were treated under the same conditions with undifferentiated 3T3-L1 cells or without 3T3-L1 adipocytes. Total content of insulin in MIN6 cells was obtained by lysing cells with lysis buffer (PBS containing 0.1% Triton X100) after washing cells twice with PBS.

Table 1 The sequences of primers used in the experiments

Name	GenBank No.	Product size	Sequences	Annealing temperature	Cycles
Insulin	NM-008387	122	F: 5′-CAC CCC ACC CAG GCT TTT G-3′ R: 5′-TCC TCC ACT TCA CGG CGG-3′	52	19
Glucokinase	L38990	162	F: 5′-CAC CCA ACT GCG AAA TCA CC-3′ R: 5′-CAT TTG TGG GGT GTG GAG TC-3′	56	30
UCP-2	U69135	255	F: 5′-CCT ACA GAT GTG GTA AAG GTC CGC-3′ R: 5′-AGA AGT GAA GTG GCA AGG GAG G-3′	56	28
Kir6.2	D50581	261	F: 5′-TCG TGT CCA AGA AAG GCA ACT G-3′ R: 5′-GGA AGG CAG ATG AAA AGG AGT GG-3′	56	28
18S	AF-372731	187	F: 5′-CGG CTA CCA CAT CCA AGG AA-3′ R: 5′-GCT GGA ATT ACC GCG GCT-3′	60	15
TNF-α	NM-013693	286	F: 5′-AGC CAG GAG GGA GAA CAG AAA C-3′ R: 5′-TCA GTA GAC AGA AGA GCG TGG TGG-3′	56	30
Leptin	NM-009493	212	F: 5′-TCC AGA AAG TCC AGG ATG ACA CC-3′ R: 5′-CAC ATT TTG GGA AGG CAG GC-3′	62	30
Resistin	NM-022984	300	F: 5-ACC TTT CAT TTC CCC TCC TT-3′ R: 5-TCC AGT CTA TCC TTG CAC AC-3	53	28
Adiponectin	NMU49915	322	F: 5-TTC CTC TTA ATC CTG CCC AG-3′ R: 5-CGA TAC ACA TAA GCG GCT TC-3′	53	30

Tyrosine kinase activity assay

MIN6 cells were lysed in protein extraction buffer (6% sodium dodecyl sulfate (SDS), 0.14 M Tris, pH = 6.8, containing 22.8% glycerol). Homogenized cell lysates were then centrifuged at 4°C for 15 min. The supernatant was collected and protein concentration was determined. Equal amounts of protein were loaded and separated by electrophoresis with pre-stained molecular weight standards and a positive control (EGF stimulated A431 cell lysate). The gel was then blotted onto a nitrocellulose membrane (Biorad). The membrane was blocked for 1 h at room temperature in Tris-Buffered saline (TBS)-Tween buffer (0.05 M Tris-Base, concentrated HCl, 1.3 M NaCl and 0.1% Tween-20) supplemented with 5% (w/v) fat-free milk, and incubated overnight at 4°C with mouse monoclonal anti-phosphotyrosine antibody (diluted at 1:1000). The blot was then washed three times in TBS-Tween buffer and incubated for 1 h at room temperature with a horseradish peroxidase conjugated anti mouse IgG (from sheep) at 1:2000, and visualized using the Immunostar HRP chemiluminescent kit (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis

Results are shown as mean values ± SEM. Statistical differences were analyzed by one-way ANOVA followed by the Dunnett post hoc test. Data for percent changes were analyzed using the Kruskal–Wallis H-test. $P < 0.05$ was considered to be significantly different. All experiments conformed to the Australian NH&MRC ethics code of practice.

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