Influence of Human Insulin and Insulin Analogues on the Expression of Insig-2 mRNA in 3T3-L1 Adipocytes

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Abstract Objective: To observe the different influence on the expression of Insig-2 mRNA under the condition of human insulin and insulin analogues in 3T3-L1 Adipocytes. **Methods**: First, experiment in vitro, to culture 3T3-L1 preadipocytes in the solution, which contains human insulin or insulin analogues. The expression of insig-2 mRNA in the 3T3-L1 preadipocytes and the differentiating 3T3-L1 adipocytes was examined by RT-PCR. Second, experiment in vivo, to inject insulin analogues, including insulin glargine and detemir, into the subcutaneous tissue of Wistar Rats. One month later, all rats were dissected to examine the expression of insig-2 mRNA in adipocytes. **Results**: The expression of insig-2 mRNA in the 3T3-L1 preadipocytes and the differentiating 3T3-L1 adipocytes intervened by insulin detemir was lower than human insulin and other analogy ($P<0.05$). Also, the decrease in phenomenon would be detected in the in vivo experiment ($P<0.05$). However, there were no significant difference both lipid and glucose homeostasis in wistar rats. **CONCLUSION**: The expression of insig-2 mRNA in 3T3-L1 adipocytes could be decreased by insulin detemir.

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Key Words:3T3-L1 Cells; Adipocyte; Ditemir; Insig-2 gene

1. Introducion

Insulin-induced genes (insigs), including insig-1 and insig-2 two isoforms[1, 2] play an important role in the formation of cholesterol, FFA, phosphatides, lipid and the differentiation of adipocytes. Insig-2 had recently been implicated as a susceptibility gene in BMI, obesity[3, 4], glucose and lipid homeostasis[5], and metabolic syndrome[6] in several but not all populations. Adipocyte differentiation of 3T3-L1 cells is associated with a 13-fold increase in expression of insig-2[7]. Over-expression of insig-1 or insig-2 by transfection could decrease lipogenic enzymes to inhibit adipocytes differentiation and lipid formation[8, 9].

Human insulin and insulin analogues, as the most general clinical hypoglycemic agents, have been compared metabolic and mitogenic potencies so as to evaluate their security in clinic. Insulin-induced gene (insig), which plays an important role in the formation of cholesterol, FFA, phosphatides, lipid and the differentiation of adipocytes, is relatively novel genes and its isoforms including insig-1 and insig-2. Whether human insulin and insulin analogues have different influence on adipocytes metabolism or not is unknown. This experiment is designed to find the differences. During the experiment, 3T3-L1 cells is cultured in culture medium including human insulin or insulin analogues in vitro environment and different insulin analogues are injected to Wistar rats through subcutaneous injections in vivo, then, to examine the expression of insig-2 mRNA in adipocytes by RT-PCR technique.

2. Materials and Methods:

2.1 Materials

2.1.1 3T3-L1 preadipocytes were purchased from China Center for Type Culture Collection, Wuhan University. High glucose Dulbecco's Modified Eagle Medium(DMEM) was the product from Beijing Solarbio, Ltd. Dimethyl sulfoxide (DMSO) and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma, USA. Trizol reagent was purchased from Tiangen Biotech, China. Insulin and analogues, including Human insulin(HI), Aspart(A), Lispro(L), Glargine(G) and Insulin Detemir(Da and Db), were purchased from the hospital's medicine department. RT-PCR kit was purchased from Promega, USA. Wistar rats were purchased from Experimental Animal Center of Zhenzhou University, China. The primers were synthesized by Invitrogen, USA.

2.2 Methods

2.2.1 Experiment in vitro: 3T3-L1 cell culture and differention

3T3-L1 preadipocytes were cultured in DMEM media supplemented with 10% fetal bovine serum (FBS) in 5% CO_2 at 37℃. The cells, were divided into

six groups depending on Human insulin and Anagolues, and were differentiated into mature adipocytes by the designed procedures. Briefly, two-day post-confluent cells (day 1) were supplemented with 10%FBS + 0.5 mmol/L IBMX + 0.5μmol/L DEX + INS (6 groups, including 1.72μmol/L(284U/L) HI, A, L, G, and 1.72μmol/L (71.1U/L) Da and 284U/L Db)for two days. Then, the cells were kept respectively for two more days in culture medium with 10%FBS + INS (the same as above). And, in the latter eight days, the cells were cultured in fresh medium with 10%FBS and refresh the medium in every two days. Meanwhile, Oil Red O staining was performed to identify adipocytes at the day 4, 8 and 12. Then the Oil Red O dye in mature adipocytes was extracted by isopropanol and its optical density (OD) value was examined by spectrophotometer and was used to reflect the fat content indirectly in cells.

3T3-L1 preadipocytes were cultured in medium with 10%FBS + INS（including two groups, 100nmol/L HI, A, L, G and Da, and 16.5 U/L Db; 1000nmol/L HI, A, L, G and Da, and 165 U/L Db） for 24 hours.

2.2.2 Experiment in vivo: Animals

Due to the characteristic of nighttime feeding and the instant hypoglycemic effect of human insulin, Aspart and Lispro, we just utilized Glargine and Insulin Detemir in order to keep the steady blood drug concentration and reduce injection time.

Forty-five wistar rats, which were raised by high-fat and sugar diet, were divided into three groups designated the control group, the Glargine-injected group and the insulin Detemir group. The rats in control group were injected Normal saline (NS), and the rats in the other groups were injected Glargine (G) and Insulin Detemir (D) separately. The rats in each group were injected the respective insulin for thirty days. In the former seven days, the injected insulin dose was increased gradually from 1 unit to 6 units, and the insulin dose was maintained at 6 units per day in the latter days. One month later, all rats were dissected to examine the expression of insig-2 mRNA in adipocytes and to measure serum cholesterol (CHO), triglyceride (TG), LDL-cholesterol (LDL-c) and HDL-cholesterol (HDL-c).

2.2.3 RNA isolation and RT-PCR amplification

3T3-L1 differentiating cells at the indicated time points (0,4,8,12 days), 3T3-L1 preadipocytes and rats'adipocytes were harvested by adding Trizol reagent to the culture plate. According to the Trizol reagent directions, total cellular RNA was isolated by spectrophotometry by A_{260}/A_{280}=1.8-2.0. Primers included insig-2 sense primer: 5'- TGG CGG AAG GAG AGA CGG AG-3', antisense primer :5'- CGT CTG CCC TCT TCA TTC TTG -3',length: 680bp. And β-actin sense primer: 5'- ATC ATG TTT GAG ACC TTC AAC A -3', antisense primer : 5'- CAT CTC TTG CTC GAA GTC CA -3',length: 318bp. The volume of reaction mixture for RT was 20μL and for PCR was 25μL. For RT-PCR, 3μL RNA was reverse-transcribed with standard reagents. The cDNA was amplified by PCR under the following protocol: 2 minutes at 95℃, 30 seconds at 95℃, 30 seconds at 51℃, 1 minute at 72℃ for 35 cycles, followed by 5-minute extension at 72℃. After PCR amplification, aliquots of PCR products were separated by 2.0% agarose gel electrophoresis, with the standard DNA markers scanned and analyzed by densitometry.

2.3 Statistical analysis

The results were expressed as means±standard deviation ($\bar{x}\pm$S).For comparison of the differences among the groups, analysis of variance (one-way ANOVA) was used. And Student's t tests were conducted when comparing mean levels between two groups. A P value less than 0.05 was considered statistically significant.

3. Resulus

3.1The expression of insig-2 mRNA in 3T3-L1 preadipocytes intervened by human insulin and insulin analogues (Fig. 1).

No matter the insulin concentration in medium was 100nmol/L or 1000nmol/L, the expression of insig-2 mRNA was downregulated obviously ($P<0.05$). It was obvious that the expression of insig-2 mRNA intervened by insulin detemir was lower than by human insulin and other analogues in both groups (fig 1; $P<0.02$ in 100 nmol/L and $P<0.04$ in 1000nmol/L). Compared with the groups of 100nmol/L insulin, the expression of insig-2 mRNA was upregulated under the condition of the same insulin in the groups of 1000nmol/L insulin ($P<0.05$).

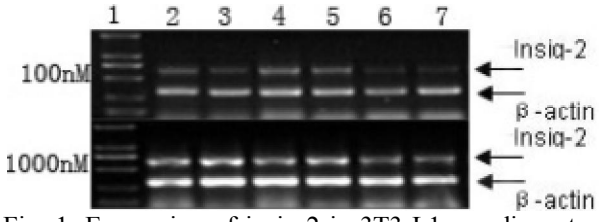


Fig. 1. Expression of insig-2 in 3T3-L1 preadipocytes cultured in the medium including 100nmol/L and 1000nmol/L insulin concentration. Lane 1: 1200-bp DNA marker; Lane 2: novolin; Lane 3: aspart; Lane 4: lispro; Lane 5: glargine; Lane 6: detemir A; Lane 7: detemir B.

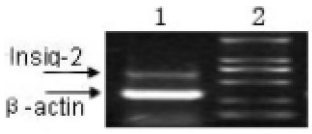
In addition, there was no significant difference in the insig-2 mRNA between detemir A and B in the same groups (fig 1;$P>0.05$). (fig 1; mean value ± SD; 100nM:HI, 0.58±0.16; A, 0.40±0.13; L, 0.69±0.14; G,

0.58±0.14; Da, 0.24±0.06; Db, 0.20±0.07; 1000nM: HI, 0.87±0.21; A, 0.89±0.21; L, 0.73±0.18; G, 0.93±0.16; Da, 0.62±0.14; Db, 0.59±0.14)

3.2. The expression of insig-2 mRNA in the differentiating adipocytes intervened by human insulin and insulin analogues (Fig. 2).

With the extension of differentiating days, the expression of insig-2 mRNA in the 3T3-L1 cells was upregulated. During the whole differentiating procedure, the expression of insig-2 mRNA intervened by insulin detemir was lower than human insulin and other analogues ($P<0.05$), except for its low level expression in lispro groups in fourth day ($P<0.05$).

A:



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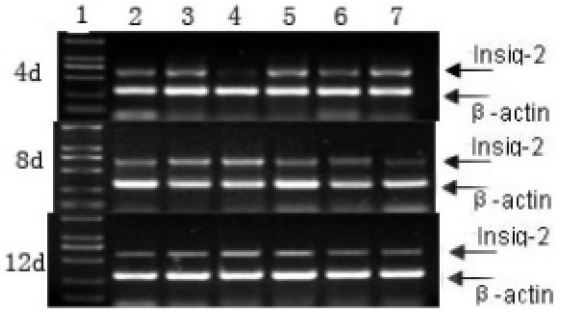

Fig. 2. Expression of insig-2 in 3T3-L1 cells during differentiation. A: day 0; Lane 1: no interfere group; Lane 2: 1200-bp DNA marker. B: the indicated days (4, 8, 12); Lane 1: 1200-bp DNA marker; Lane 2: novolin; Lane 3: aspart; Lane 4: lispro; Lane 5:glargine; Lane 6: detemir A; Lane 7: detemir B.

In the differentiating 3T3-L1 cells, the expression of insig-2 mRNA was upregulated during the differentiating procedure (fig 2;$P < 0.05$). The expression of insig-2 mRNA intervened by insulin detemir was lower than human insulin and other analogues during the differentiating procedure (fig 2;$P<0.05$), expect the lower expression in lispro group in the day 4 (fig 2;$P<0.01$). Nevertheless the expression of insig-2 mRNA in lispro group was elevated in the day 8 and 12(fig 2;$P<0.05$). (fig 2; mean value±SD; 0d:HI, 0.15±0.08; A, 0.15±0.08; L, 0.15±0.08; G, 0.15±0.08; Da, 0.15±0.08; Db, 0.15±0.08; 4d: HI,0.43±0.10; A, 0.47±0.09; L, 0.19±0.05; G, 0.49±0.10; Da, 0.38±0.07; Db, 0.35±0.07; 8d: HI,0.61±0.17; A, 0.68±0.12; L, 0.73±0.10; G, 0.71±0.19; Da, 0.56±0.09; Db, 0.45±0.13; 12d: HI,0.81±0.24; A, 0.79±0.20; L, 0.81±0.18; G, 0.79±0.19; Da, 0.73±0.10; Db, 0.60±0.12)

3.3 Insulin detemir up-regulated the content of lipid in differentiated adipocytes

The higher OD value in detemir B groups compared with the others indicated that the adipocyte in detemir B was possibly more mature than in the other groups (fig 3; OD, mean value±SD; HI, 0.799±0.076; A, 0.81±0.082; L, 0.793±0.064; G, 0.789±0.141; Da, 0.772±0.092; Db, 0.878±0.101*; *significantly different from the other group, $P<0.05$).

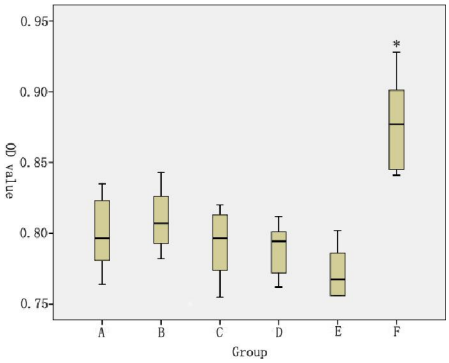

Fig. 3. OD value of Oil Red O dye in mature adipocytes. Column A: novolin; Column B: aspart; Column C: lispro; Column D: glargine; Column E: detemir A; Column F: detemir B. *P<0.05 vs novolin and another analogues.

3.4. The expression of insig-2 mRNA in rats adipose tissue intervened by both Glargine and Insulin detemir (Fig. 4).

The expression of insig-2 mRNA in rats adipose tissue intervened by Insulin detemir was lower than normal saline(control group) and Glargine (fig 4; $P<0.02$). However, The expression of insig-2 mRNA in rats adipose tissue was no statistically significant difference between the normal saline group and glargine group(fig 3;$P>0.05$). (fig 4; mean value±SD: control groups,0.84±0.11; G,0.81±0.12; D,0.43±0.0 4)

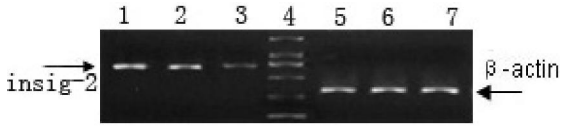

Fig. 4. Expression of insig-2 in rats' adipose tissue. Lane 1 and 5: control group; Lane 2 and 6: glargine; Lane 3 and 7: detemir; lane 4: 1200-bp DNA marker.

3.5. There is not influence on serum lipid in the vivo;

There was no statistically significant difference on lipid, concerned serum CHO, TG, LDL-c, and HDL-c, among groups intervened by normal saline, glargine and detemir.(fig 5;$P>0.05$). (fig 5; mean value±SD;

CHO: NG 1.99±0.41,G 1.93±0.23,D 2.01±0.21;TG: NG 1.17 ± 0.27,G 1.22 ± 0.21,D 1.21 ± 0.13; LDL-c:NG 0.45 ± 0.09,G 0.42 ± 0.06,D 0.47 ± 0.07;HDL-c:NG 0.55±0.09,G 0.49±0.08,D 0.47± 0.09)

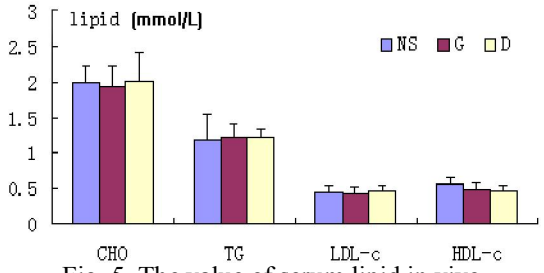

Fig. 5. The value of serum lipid in vivo.

4. Discussion

In mammalian cells, SREBPs can activate genetic transcription of key enzyme in the synthesis of lipid including cholesterol, fatty acids, phospholipids and TG[10], and recent studies have shown that they link lipid metabolism to cell growth and survival through the direct activation of additional key target genes of other cellular processes[11]. 3-hydroxy-3-methylglutaric acid coenzyme A reductase（HMGR） is the reductase of cholesterol synthesis. Insulin-induced genes (Insigs), as the novel gene discovered in the recent years, contain two isoforms: Insig-1 and Insig-2[1,2]. The former is highly expressed in the liver, whereas the latter is ubiquitously expressed, such as adipose tissue, muscles, skin and liver[12]. Insigs can influence lipid metabolism in the two followed ways.

Due to the up-regulation of cholesterol in cells, Insigs can mediate lipid metabolism through binding to tsterol-sensing domain in SREBPs and HMGR[13]. Side-chain oxysterols are thought to signal excess cholesterol by binding to Insigs[14]. First, when insig proteins bind to the SCAP-SREBP complex in endoplasmic reticulum(ER), it forms the INSIG-SCAP-SREBP complex. COP Ⅱ proteins can no longer bind to SCAP(SREBP cleavage-activating protein), and then inhibit the SREBP(sterol regulatory element-binding proteins) moving to Golgi and the release of nuclear protein[15, 16]. Hence it promotes the ER retention of SCAP-SREBP and inhibit its shift and the processing in Golgi[12, 15].Second, when insig proteins combine with HMGR, the synthesis of cholesterol is inhibited by promoting reductase ubiquitination/degradation and down-regulating mevalonate[13,16]. Excess sterols cause the reductase to bind to ubiquitin ligases gp78 and Trc8[17]. Reductase-Trc8 binding is mediated by both Insig-1 and Insig-2, whereas reductase-gp78 binding is mediated primarily by Insig-1[18].

In Golgi ,the nuclear protein derivated from SREBPs can combine with the promoter and the enhancer of target gene, further to activate and synthesize the rate-limiting enzymes which are necessary for synthesis of free fatty acids, phospholipids, triglycerides, and cholesterol[19,20]. The low concentration of cholesterol in cells can prevent insig proteins separate from SCAP[2,20]. When the concentration of cholesterol is higher in cells, the phenomenon is reverse. SREBP-1c regulates mainly the gene activations of fatty acids and triglycerides; the target genes contain low density lipoprotein receptor, acetyl coenzyme A carboxylase, fatty acid synthetase, glucokinase, phosphoenolpyruvate carboxykinase et al[21, 22]. SREBP-2 is involved in the activation of cholesterol[21]. Due to the existence of peroxisome proliferator-activated receptor gamma (PPARγ), Insig-1 can be influenced by PPAR-γ agonist[23]. Troglitazone, a synthetic agonist of PPAR γ , induces a marked recruitment of HMGR to Insig-2[24]. Additionally, the scavenger receptor CD36 increases the expression of Insig-1 and Insig-2 gene through activation of nuclear receptor PPAR γ [24].

In liver cell, the concentration of cholesterol in cells has a primary influence on the expression of insig-1, but not on insig-2. The latter has negative correlation with serum insulin[25]. Lee etal. reported that when the cholesterol was insufficient, the decrease rate of insig-1 was more than fifteen times than insig-2[25]. However it is not clear that what factor plays a major role in the expression of insig-2 in adipose tissue. Guenther Boden reported that insulin stimulated activation of SREBP-1c in the liver, at least in part, by suppressing INSIG-1 and -2, whereas in adipose tissue, an increase in INSIG-1 and -2 prevented SREBP-1c activation[26]. It was all-known that insig-2, which had a high increase in the differentiating adipocytes, participated in the differentiation of adipose cell and the formation of lipid[7]. Over-expression of insig-1 or insig-2 by transfection could decrease lipogenic enzymes to inhibit adipocytes differentiation and lipid formation[8, 9]. Moreover, in the 3T3-L1 cells cultured in low-glucose medium, increasing insig-2 led to relative inhibit of adipocytes differentiation and lipid formation[8].

Recently, several studies indicated that the genes encoding phosphoenolpyruvate carboxykinase(PCK2)[27,28] and glucokinase [29], two key enzymes in gluconeogenesis and glycolysis were SREBP-target genes. Insig-2 reduces the proteolytic activation of the membrane-bound SREBP precursor[30]. Additionally, Insig-2 had recently been implicated as a susceptibility gene in BMI, obesity[3,4], glucose and lipid homeostasis[5], and metabolic syndrome[6] in several but not all populations. It was reported that the INSIG2 gene was associated with metabolic syndrome (MetS) in patients treated with atypical antipsychotics

(AAPs) independently or in an interactive manner with INSIG1[31].

In our study, we found that insig-2 was up-regulated with the extention of the days in the 3T3-L1 differentiating adipocytes. It is consistent with that of previous studies [8,9]. Moreover, the expression of insig-2 interfered by insulin detemir was obviously lower and the lipid in corresponding cells was more than by human insulin and the other analogues. With the increase of insulin in medium， insig-2 was increased gradually in 3T3-L1 preadipocytes. The phenomenon of relative insig-2 decrease influenced by insulin detemir was observed in vitro cultured cells and in vivo adipose tissue. However serum lipid was normal. The possible explanation was that rats, not diabetic rats, involved in this experiment with the normal mechanism were able to maintain the lipid homostasis. Accordingly, we concluded that in comparison with human insulin and the other insulin analogues, insulin detemir could relatively induce insig-2 to be down-regulated.

Whether human insulin and insulin analogues has different effect on the expression of insig-1 in adipocytes and on insig-1 and insig-2 in other tissues is unclear. Due to the complication of serum lipid metabolism and lipometabolism, the result of experiment in vitro cannot represent the influence by insulin detemir on serum lipid, adipocyte differentiation and adipose formation. The further studies should be focused on the mechanism of dyslipidemia and the relationships among insigs, different clinical insulin and lipid metabolism in diabetes.

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