

## Research Paper

## Involvement of ATP-sensitive potassium channels in proliferation and differentiation of rat preadipocytes

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**Abstract:** This paper was aimed to investigate the effects of ATP-sensitive potassium channels on the proliferation and differentiation of rat preadipocytes. We examined the expression of sulphonylurea receptor 2 (SUR2) mRNA in preadipocytes and adipocytes obtained by inducing for 5 d and the effects of the inhibitor (glibenclamide) and opener (diazoxide) of ATP-sensitive potassium channels on the expression of SUR2 mRNA in preadipocytes by real-time PCR. Preadipocyte proliferation and cell cycle were measured by MTT spectrophotometry and flow cytometer. The content of intracellular lipid was measured by oil red O staining, cell diameter was determined by Image-Pro Plus 5.0 software and the expression of peroxisome proliferator-activated receptor- $\gamma$  (PPAR- $\gamma$ ) mRNA was estimated by RT-PCR. SUR2 mRNA was expressed in both preadipocytes and adipocytes obtained by inducing for 5 d, and the expression in adipocytes was obviously higher than that in preadipocytes. Glibenclamide inhibited the expression of SUR2 mRNA in preadipocyte, promoted preadipocyte proliferation in a dose-dependent manner, increased the cell percentages in G<sub>2</sub>/M + S phase, increased lipid content, augmented adipocyte diameter, and promoted the expression of PPAR- $\gamma$  mRNA. But the actions of diazoxide were contrary to those of glibenclamide. These results suggest that ATP-sensitive potassium channels regulate the proliferation and differentiation of preadipocytes, and PPAR- $\gamma$  is probably involved in the effect of ATP-sensitive potassium channels.

**Key words:** rat; potassium channels; sulphonylurea receptor; adipocyte; obesity; cell proliferation; cell differentiation

## ATP 敏感钾通道参与大鼠前脂肪细胞增殖和分化

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**摘要:** 为了探讨 ATP 敏感钾通道在前脂肪细胞增殖分化中作用, 本实验用逆转录实时定量 PCR 方法检测大鼠前脂肪细胞和诱导 5 d 获得的脂肪细胞中该通道磺脲类受体 2 (sulphonylurea receptor 2, SUR2) mRNA 表达, 探讨该通道阻滞剂格列本脲和激动剂二氮嗪对前脂肪细胞中 SUR2 mRNA 表达的影响; MTT 检测前脂肪细胞增殖; 流式细胞仪检测细胞周期; 油红 O 染色法检测细胞内脂质含量; Image-Pro Plus 5.0 软件测量细胞直径; 逆转录 PCR 检测过氧化物酶体增殖物激活受体- $\gamma$  (peroxisome proliferator-activated receptor- $\gamma$ , PPAR- $\gamma$ ) mRNA 表达。结果显示: 前脂肪细胞及诱导 5 d 获得的脂肪细胞均有 SUR2 mRNA 表达, 且后者明显高于前者; 格列本脲抑制前脂肪细胞 SUR2 mRNA 表达, 剂量依赖性地促进前脂肪细胞增殖, 增加 G<sub>2</sub>/M + S 期细胞百分比, 增加细胞脂质含量, 使脂肪细胞直径增大, 增加 PPAR- $\gamma$  mRNA 的表达; 二氮嗪在这些方面的作用与格列本脲相反。以上结果提示, ATP 敏感钾通道在前脂肪细胞增殖和分化中可能起调节作用, PPAR- $\gamma$  可能参与这些作用。

**关键词:** 大鼠; 钾通道; 磺脲类受体; 脂肪细胞; 肥胖症; 细胞增殖; 细胞分化

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Obesity is a status of excessive body fat deposition with a body mass index of 30 or above. Although it is not a kind of disease, it increases the possibility to develop diabetes, hypertension or cardiovascular diseases. In the last two decades, obesity has become a major health risk in most parts of the world. The pathogenesis has not been thoroughly elucidated. ATP-sensitive potassium channels widely exist in various cells. The channels link the electrical activity of cell membranes to cellular metabolism. It has been shown that these channels are involved in the proliferation of vessel smooth muscle cells<sup>[1]</sup>. Gabrielson *et al.*<sup>[2,3]</sup> have reported that adipocytes express ATP-sensitive potassium channels, and its receptor is sulfonylurea receptor 2 (SUR2). Alemzadeh *et al.*<sup>[4,5]</sup> have reported that diazoxide, an opener of ATP-sensitive channels, exerts an antiobesity effect in obese Zucker rats. Besides, they recently reported that diazoxide exerted a significant antiobesity effect in hyperinsulinemic obese adults<sup>[6]</sup>. However, there is limited information available about the role of ATP-sensitive channels in the proliferation and differentiation of preadipocytes. The aim of this research was to explore the effects of ATP-sensitive potassium channels on the proliferation and differentiation of preadipocytes.

## 1 MATERIALS AND METHODS

Reagent: DMEM, DMEM/F12 medium, newborn calf serum, insulin, transferrin, triiodothyronine (T3), collagenase, oil red O, RNase, glibenclamide, diazoxide were purchased from Sigma Company. Propidium iodide (PI) was purchased from Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd. RevertAid™ First Strand cDNA Synthesis Kit was a product from Fermentas Company. SYBR Green Real-time PCR Master Mix was from Toyobo Company.

Main instruments: Rotor-Gene RG-3000 (Cobett Company, Australia), Laber-Centrifuge (Sigma Company, Germany), FACalibration (BD Company, America)

### 1.1 *Rat primary adipocyte culture*

According to the methods of Deslex *et al.* and Liu *et al.*<sup>[7,8]</sup>, 8-week-old male Sprague-Dawley rats (from the Experimental Animal Center of Tongji Medical College) used in this study were fed with ordinary chow diet and killed by cervical dislocation. Adipose deposits (periepididymal) were resected under sterile conditions, bathed three times in phosphate-buffered saline (PBS) with antibiotics, and then freed as much as possible from blood capillaries with eye scissors under anatomy microscope. The tissues were digested for 90 min at 37 °C in DMEM containing 1 g/L

collagenase, then put into DMEM and mixed them up. The mixture was centrifugated at 1 000 r/min for 5 min. The supernatant was discarded and the sediment was suspended in DMEM. After filtrated through 250-mesh screen and centrifuged again, the sediment was resuspended in DMEM supplemented with 10% newborn bovine serum (NBS), penicillin and streptomycin, and cultured in culture flasks. The cells were induced for 5 d in serum-free DMEM/F12 medium supplemented with 5 mg/L insulin, 10 mg/L transferrin and 200 pmol/L T3 (ITT).

### 1.2 *Reverse transcription-PCR (RT-PCR) and Real-time PCR*

Total RNA was extracted according to the manufacturer's instruction (Fermentas). The RNA content was quantified by ultra violet spectrophotometer at 260 nm. One microgram aliquot of total RNA was reverse-transcribed into the first-strand complementary DNA (fs cDNA) by using oligo(dT)<sub>18</sub> primer. The fs cDNA was amplified by PCR. The PCR was carried out in a total volume of 20 µL containing 20 mmol/L Tris-HCl, 50 mmol/L KCl, 1.25 mmol/L MgCl<sub>2</sub>, 0.2 mmol/L dNTP, 0.5 mmol/L of each primer, 1 U of *Taq* DNA polymerase, 2 µL cDNA. The mRNA expression of house keeping gene, GAPDH, was considered as an internal reference. Cycle parameters were as follows: 94 °C 5 min, 35 cycles (94 °C 30 s, 53 °C or 51 °C 30 s, 72 °C 40 s), 72 °C 2 min. The ordinary PCR products were separated on a 2% goldview-stained agarose gel. Real-time PCR mixture included 10 µL SYBR Green Real-time PCR Master Mix, 0.5 µmol/L of SUR2 forward primer, 0.437 5 µmol/L of SUR2 reverse primer, 2 µL cDNA. The total volume was 20 µL. Cycle parameters were as follows: 94 °C 5 min, 40 cycles (94 °C 15 s, 51 °C 15 s, 72 °C 20 s). The adipose tissue cDNA was diluted to different concentrations in order to construct the standard curves of internal reference and SUR2. The primers were designed by Primer Premier 5.0 (Table 1).

The expression of SUR2 mRNA in preadipocytes, 5-day induced adipocytes and preadipocytes treated with the inhibitor (glibenclamide) and opener (diazoxide) were measured by real-time PCR.

Preadipocytes induced for 5 d were divided into three groups: ITT group, ITT + 100 µmol/L glibenclamide group, ITT + 100 µmol/L diazoxide group. Peroxisome proliferator-activated receptor-γ (PPAR-γ) mRNA was measured by RT-PCR in these three groups.

### 1.3 *MTT spectrophotometry*

Preadipocytes were planted into 96-well plates and divided into seven groups: DMEM group, DMEM + 50 µmol/L

Table 1. Primer designation

		Primer	Length	Annealing temperature
SUR2	Forward	5'-GGGTTGTGGCAAATCGTCTC-3'	284 bp	51°C
	Reverse	5'-GGTCTCCAAAGGGTAACAAATCA-3'		
PPAR- $\gamma$	Forward	5'-CACAATGCCATCAGGTTTGG-3'	304 bp	53°C
	Reverse	5'-CCACCTCTTTGCTCTCTCC-3'		
GAPDH	Forward	5'-CCATGTTTCGTTCATGGGTGTGAACCA-3'	251 bp	51°C
	Reverse	5'-GCCAGTAGAGGCAGGGATGATGTTC-3'		

glibenclamide group, DMEM + 100  $\mu\text{mol/L}$  glibenclamide group, DMEM + 150  $\mu\text{mol/L}$  glibenclamide group, DMEM + 50  $\mu\text{mol/L}$  diazoxide group, DMEM + 100  $\mu\text{mol/L}$  diazoxide group, DMEM + 150  $\mu\text{mol/L}$  diazoxide group. After culture for 24 h, MTT solution was added to every group, and the cells were continued to culture for 4 h. The medium was discarded, and then DMSO was added to dissolve the crystal. Ten minutes later, the absorbance at 490 nm was measured.

#### 1.4 Cell cycle examination

Primary preadipocytes were planted into cultured flasks and divided into groups as above in MTT spectrophotometry. After cultured for 24 h, the cells in all groups were digested with 0.25% trypsin and 0.02% EDTA, washed twice with PBS and fixed with 800 mL/L ice alcohol. Thirty minutes later, the cells were centrifuged and rinsed with PBS three times. The precipitation was suspended by PBS with 10 mg/L PI and 1.0 g/L RNase. After 20 min the cell cycle was measured by flow cytometer.

#### 1.5 Oil red O staining

Preadipocytes were planted into 96-well plates, divided into seven groups: ITT group, ITT + 50  $\mu\text{mol/L}$  glibenclamide group, ITT + 100  $\mu\text{mol/L}$  glibenclamide group, ITT + 150  $\mu\text{mol/L}$  glibenclamide group, ITT + 50  $\mu\text{mol/L}$  diazoxide group, ITT + 100  $\mu\text{mol/L}$  diazoxide group, ITT + 150  $\mu\text{mol/L}$  diazoxide group. After induced for 5 d, the cells were washed carefully with PBS, fixed with 10% formalin solution for 2 h at room temperature. After fixation, the cells were washed twice with PBS and stained by 4.2% oil red O in isopropanol for 1 h, and then washed and extracted by isopropanol. Finally the absorbance at 510 nm was measured.

#### 1.6 Cell diameter determination

Preadipocytes were planted into 96-well plates, divided into three groups: ITT group, ITT + 100  $\mu\text{mol/L}$  glibenclamide group, ITT + 100  $\mu\text{mol/L}$  diazoxide group. After induced for 5 d, the cells were digested and taken pictures. The cell diameter was determined by Image-Pro Plus software.

#### 1.7 Statistical analysis

All data were expressed as mean $\pm$ SD. Data were evaluated for statistical significance by analysis of variance or chi-square test.

## 2 RESULTS

### 2.1 Expression of SUR2 mRNA in preadipocytes and adipocytes

SUR2 mRNA expression in preadipocytes and adipocytes induced for 5 d was measured by real-time PCR ( $n=3$ ). SUR2 mRNA in adipocytes was increased by 36.67 times compared with that in preadipocytes ( $P<0.01$ ) (data not shown).

### 2.2 Effects of glibenclamide and diazoxide on SUR2 mRNA expression in preadipocytes

After preadipocytes were treated with glibenclamide and diazoxide for 24 h, SUR2 mRNA expression in preadipocytes was measured by real-time PCR ( $n=3$ ). SUR2 mRNA in preadipocytes treated with glibenclamide was decreased by 0.23 times compared with that in the control group ( $P<0.01$ ). SUR2 mRNA in preadipocytes treated with diazoxide was increased by 54.57 times compared with that in the control group ( $P<0.01$ ) (data not shown).

### 2.3 Effects of glibenclamide and diazoxide on the proliferation of preadipocytes

#### 2.3.1 MTT

The  $A_{490}$  value increased with the increase of glibenclamide concentration. The  $A_{490}$  value in all glibenclamide groups was higher than that in the control group ( $P<0.01$ ). But the  $A_{490}$  value decreased with the increase of diazoxide concentration. The  $A_{490}$  value in all diazoxide groups was lower than that in the control group ( $P<0.01$  or  $P<0.05$ ) (Fig.1). The effects of glibenclamide and diazoxide were in a dose-dependent manner.

#### 2.3.2 Flow cytometer

After the cells were treated with glibenclamide and diazoxide for 24 h, the cell percentages in  $G_2/M + S$  phases increased

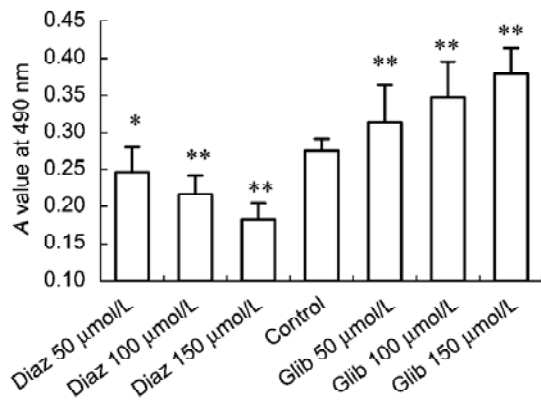


Fig. 1. Effects of different concentrations of glibenclamide and diazoxide on the cell proliferation measured by MTT. \* $P<0.05$ , \*\* $P<0.01$  vs the control group. Glib, glibenclamide; Diaz, diazoxide.  $n=12$ .

in glibenclamide group, while the cell percentages in  $G_2/M + S$  phases decreased in diazoxide group ( $P<0.01$  or  $P<0.05$ ) (Table 2).

**2.4 Effects of glibenclamide and diazoxide on the differentiation of preadipocytes**

After the cells were induced for 5 d, they were stained by oil red O and extracted by isopropanol. The  $A_{510}$  value in all glibenclamide groups was higher than that in the control group ( $P<0.05$ ). Compared with that in the control group, the  $A_{510}$  value in all diazoxide groups was lower ( $P<0.05$ ), but there were no differences among all glibenclamide groups and all diazoxide groups (Table 3).

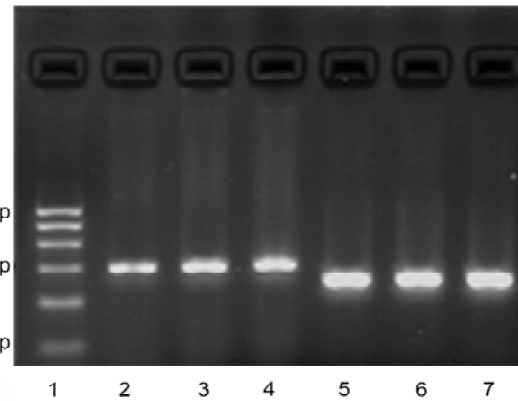


Fig. 2. Effects of glibenclamide and diazoxide on the expression of PPAR- $\gamma$  mRNA. 1, Marker; 2, PPAR- $\gamma$  mRNA in 100  $\mu\text{mol/L}$  diazoxide group; 3, PPAR- $\gamma$  mRNA in the control group; 4, PPAR- $\gamma$  mRNA in 100  $\mu\text{mol/L}$  glibenclamide group; 5, GAPDH mRNA in 100  $\mu\text{mol/L}$  diazoxide group; 6, GAPDH mRNA in the control group; 7, GAPDH mRNA in 100  $\mu\text{mol/L}$  glibenclamide group.

The cell diameter was measured by Image-Pro Plus software. The diameter of the cell in 100  $\mu\text{mol/L}$  glibenclamide group was bigger than that in the control group ( $P<0.05$ ), while the diameter of the cell treated with 100  $\mu\text{mol/L}$  diazoxide was smaller ( $P<0.05$ ) (Table 4).

Photoes were taken after PCR product was electrophoresed (Fig.2). The photoes were analyzed by Gel-pro Analyzer. The relative expression of PPAR- $\gamma$ /GAPDH in 100  $\mu\text{mol/L}$  diazoxide group was decreased by 0.869 times compared with that in the control group. However, the relative ex-

Table 2. Effects of different concentrations of glibenclamide and diazoxide on the cell cycle measured by flow cytometer (%)

	Control	Glibenclamide ( $\mu\text{mol/L}$ )			Diazoxide ( $\mu\text{mol/L}$ )		
		50	100	150	50	100	150
$G_0/G_1$	69.25 $\pm$ 2.97	65.47 $\pm$ 2.07	59.63 $\pm$ 2.58	50.41 $\pm$ 1.34	73.13 $\pm$ 3.01	76.93 $\pm$ 1.95	79.02 $\pm$ 2.48
$G_2/M + S$	30.75 $\pm$ 1.84	34.53 $\pm$ 1.89*	40.37 $\pm$ 2.73**	49.59 $\pm$ 2.05**	26.87 $\pm$ 2.08*	23.07 $\pm$ 1.74**	20.98 $\pm$ 1.54**

mean $\pm$ SD,  $n=3$ . \* $P<0.05$ , \*\* $P<0.01$  vs the control group.

Table 3. Effects of different concentrations of glibenclamide and diazoxide on the content of lipid

	Control	Glibenclamide ( $\mu\text{mol/L}$ )			Diazoxide ( $\mu\text{mol/L}$ )		
		50	100	150	50	100	150
$A_{510}$ value	0.187 $\pm$ 0.022	0.249 $\pm$ 0.060*	0.247 $\pm$ 0.040*	0.264 $\pm$ 0.018*	0.154 $\pm$ 0.013*	0.155 $\pm$ 0.015*	0.159 $\pm$ 0.014*

mean $\pm$ SD,  $n=8$ . A values at 510 nm were measured by oil red O staining and isopropanol extracting. \* $P<0.05$  vs the control group.

Table 4. Effects of glibenclamide and diazoxide on the diameter of cells ( $\mu\text{m}$ )

	Diazoxide 100 $\mu\text{mol/L}$	Control	Glibenclamide 100 $\mu\text{mol/L}$
Diameter of cells	22.550 $\pm$ 4.306*	26.474 $\pm$ 3.286	31.552 $\pm$ 4.614**

mean $\pm$ SD,  $n=700$ . \* $P<0.01$  vs the control group, # $P<0.01$  vs 100  $\mu\text{mol/L}$  diazoxide group.

pression of PPAR- $\gamma$ /GAPDH in 100  $\mu\text{mol/L}$  glibenclamide group was increased by 1.158 times compared with that in the control group. PPAR- $\gamma$  mRNA expression in 100  $\mu\text{mol/L}$  diazoxide group was higher than that in the control group ( $P < 0.05$ ). But PPAR- $\gamma$  mRNA expression in 100  $\mu\text{mol/L}$  glibenclamide group was lower than that in the control group ( $P < 0.05$ ).

### 3 DISCUSSION

We confirm that SUR2 mRNA is expressed both in preadipocytes and adipocytes obtained by inducing for 5 d. It is in coincidence with report from Gabrielson *et al*<sup>[2]</sup>. Moreover, adipocytes obtained by inducing for 5 d express more SUR2 mRNA than preadipocytes.

After treatment with glibenclamide, the inhibitor of ATP-sensitive potassium channels, the expression of SUR2 mRNA in preadipocytes decreased obviously. In contrast to the role of glibenclamide, it has been found that the expression of SUR2 mRNA in preadipocytes increased markedly after treatment with diazoxide, the opener of ATP-sensitive potassium channels<sup>[9]</sup>. MTT results indicated that glibenclamide decreased the  $A_{490}$  value in a dose-dependent manner, whereas diazoxide increased the  $A_{490}$  value in a dose-dependent manner. In addition, the cell cycle measured by flow cytometer showed that glibenclamide increased the cell percentages in  $G_2/M + S$  phase, while diazoxide decreased the cell percentages in  $G_2/M + S$  phase. These results suggest that when ATP-sensitive potassium channel expression decreases, preadipocyte proliferation is promoted, but when the expression of the channels increases, preadipocyte proliferation is inhibited. That means ATP-sensitive potassium channels play a role in regulating the preadipocyte proliferation. The  $A_{510}$  value by oil red O staining reflects lipid content in the cells. The  $A_{510}$  value in all glibenclamide groups was higher than that in the control group, but the  $A_{510}$  value in all diazoxide groups was lower. The cell diameter in 100  $\mu\text{mol/L}$  glibenclamide group was bigger than that in the control group, while the cell diameter in 100  $\mu\text{mol/L}$  diazoxide group was smaller. These results indicate that when ATP-sensitive potassium channel expression decreases, preadipocyte differentiation is promoted, but when ATP-sensitive potassium channels expression increases, preadipocyte differentiation is inhibited. ATP-sensitive potassium channels might play a role in regulating preadipocyte differentiation.

PPAR- $\gamma$  belongs to orphan receptor family. PPAR- $\gamma$  plays a role in regulating cell differentiation and growth<sup>[10]</sup>. This

research showed that PPAR- $\gamma$  mRNA expression in 100  $\mu\text{mol/L}$  glibenclamide group increased, but the expression in 100  $\mu\text{mol/L}$  diazoxide group decreased. That ATP-sensitive potassium channels promote or inhibit proliferation and differentiation of preadipocytes may be related to the increase or decrease of PPAR- $\gamma$  mRNA. But it needs further study.

ATP-sensitive potassium channels participate in the proliferation and differentiation of the preadipocytes. The role possibly has relation to PPAR- $\gamma$  mRNA expression. It is to be confirmed whether the development of obesity has relation to excessive inhibition of ATP-sensitive potassium channels in preadipocytes or not. This article brings a new idea about pathogenesis elucidation and therapy of obesity.

### REFERENCES

- 1 Mu YL, Jin WB, Cai JQ, Guan FL. Effect of calcitonin gene-related peptide on the proliferation of vascular smooth muscle cells of diabetic rats. *Chin J Pharmacol Toxicol (中国药理学与毒理学杂志)* 2003; 17(5): 345-349.
- 2 Gabrielson BG, Karlsson AC, Lonn M, Olofsson LE, Johansson JM, Torgerson JS, Sjoström L, Carlsson B, Eden S, Carlsson LM. Molecular characterization of a local sulfonylurea system in human adipose tissue. *Mol Cell Biochem* 2004; 258: 65-71.
- 3 Gabrielson BL, Carlsson B, Carlsson LM. Partial genome scale analysis of gene expression in human adipose tissue using DNA array. *Obes Res* 2000; 8: 374-384.
- 4 Alemzadeh R, Slonim AE, Zdanowicz MM, Maturo J. Modification of insulin resistance by diazoxide in obese Zucker rats. *Endocrinology* 1993; 133: 705-712.
- 5 Alemzadeh R, Jacobs AW, Pitukcheewong P. Antiobesity effect of diazoxide in obese Zucker rats. *Metabolism* 1996; 45: 334-341.
- 6 Alemzadeh R, Langley G, Upchurch L, Smith P, Slonim AE. Beneficial effect of diazoxide in obese hyperinsulinemic adults. *J Clin Endocr Metab* 1998; 83: 1911-1915.
- 7 Deslex S, Negrel R, Ailhaud G. Development of a chemically defined serum-free medium for differentiation of fat adipose precursor cells. *Exp Cell Res* 1987; 168: 15-30.
- 8 Liu L (刘垒), Liu SY. Effect of compound poria prescription on proliferation and differentiation and expression of PPAR- $\gamma$  mRNA in rat preadipocytes. *Chin J Pathophysiol (中国病理生理杂志)* 2005; 21(7): 1364-1367 (Chinese, English abstract).
- 9 Babenko AP, Aguilar-Bryan L, Bryan J. A view of sur/kir6.x,  $K_{ATP}$  channels. *Annu Rev Physiol* 1998; 60: 667-687.
- 10 Camp HS, Chaudhry A, Left T. A novel potent antagonist of peroxisome proliferators activated receptor- $\gamma$  blocks adipocyte differentiation but does not revert the phenotype of terminally differentiated adipocyte. *Endocrinology* 2001; 142(7): 3207-3213.