Regulation of Adiponectin Receptor 2 Expression via PPAR-α in NIT-1 Cells

Tae Woo Jung*, Myung Won Lee**,***, Yong Jik Lee#, Seon Mee Kim**, Kyoung Tae Lee##, Wan Kyunn Whang##, Hwan Ju Cheon *, Yeon Taek Jeong*, Kun Wook Chung*, Jae Min Cho*, Do Hoon Kim* and Tae Woo Jung*

* *Samsung Biomedical Institute, Seoul, Korea*
** *Department of Family Medicine, Brain Korea 21 Project Medical Science, College of Medicine, Korea University, Seoul, Korea*
*** *Department of Anatomy, College of Medicine, Korea University, Seoul, Korea*
# *Division of Clinical Research, Seoul Medical Center Research Institute, Seoul, Korea*
College of Pharmacy, Chung-Ang University, Seoul, South Korea

Abstract. Adiponectin receptors mediate the antidiabetic effects of adiponectin. Although suggested to be mainly expressed in muscle, liver, and adipocyte cells, the expression of adiponectin receptors in β cells is unclear. Given the primary involvement of this cell type in diabetes mellitus, we presently examined the expression level of adiponectin receptor 2 (AdiR2) in β cells. Expression was significantly increased under acute hyperlipidemic conditions but impaired under chronic conditions. The impaired AdiR2 expression may play a role in worsened β cell function. Clofibrate, an agonist of peroxisome proliferator-activated receptor-alpha (PPAR-α) delayed the palmitate-induced impairment of AdiR2 expression and PPAR-α; this delay was abolished by PPAR-α targeted small interfering RNA. The results suggest that AdiR2 expression is regulated by palmitate via PPAR-α.

Key words: Adiponectin receptor 2, peroxisome proliferator-activated receptor-alpha, β cells, Clofibrate, palmitate

(*Endocrine Journal* **56**: 377-382, 2009)

ADIPONECTIN is an adipokine secreted from adipocytes, which has antiatherogenic and antidiabetic effects [1]. The latter effect seems to be mediated by a stimulation of fatty acid oxidation by the activation of AMP-activated protein kinase (AMPK) [2–6] and an antiapoptotic effect of β cells. The DNA sequences and adiponectin receptor function of two seven-transmembrane proteins designated adiponectin receptor 1 and 2; (AdiR1 and AdiR2, respectively) have been reported [2, 3]. AdiR1 is mainly expressed in skeletal muscle, whereas AdiR2 is dominantly expressed in the liver. AdiR1 and AdiR2 serve as receptors for globular and full-length adiponectin and both mediate activated AMPK, fatty acid oxidation, and glucose up-take [2–6]. Free fatty acids such as palmitate lead to the development of insulin resistance, which may also provoke β cell dysfunction or death [7–10]. Thus, it has been studied about relationship of adiponectin and obesity-induced type 2 diabetes [6].

Peroxisome proliferator-activated receptor-alpha (PPAR-α) typically regulates the expression of multiple genes associated with mitochondrial and peroxisomal fatty acid oxidation as well as lipoprotein metabolism [3]. PPAR-α is expressed in many organs including primary rat β cells [11], and has been shown to activate fatty acid oxidation [12]. PPAR-α agonists including fenofibrate and ciprofibrate prevent fatty acid-induced β cell destruction [13] and improve β cell function in the insulin resistant state [14]. It has not yet been determined whether β cell expressions of AdiR1 and AdiR2 are regulated by PPAR-α under hyperlipidemic conditions.

Presently, we studied the expressions of AdiR1 and AdiR2 in the NIT-1 mouse pancreatic β cell line under

hyperlipidemic condtions, and demonstrated that induction and impairment of AdiR2 by palmitate may be involved with the PPAR-α dependent signal pathway.

Materials and methods

Culture media and reagents

NIT-1 cells were plated at a density of 2×10^5 cells/ml and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with heat inactivated 10% (v/v) fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Palmitate was purchased from Sigma-Aldrich (St. Louis, MO). Adiponectin was purchased from Pepro Tech (Rocky Hill, NJ). For AdiR2 regulation studies, NIT-1 cells were exposed to 200 μM palmitate with or without clofibrate (kindly provided by Dr. Yong Jik Lee, Seoul Medical Center, Seoul, Korea). After 0–48 h of exposure, cells were harvested for mRNA extraction. The concentrations of palmitate and clofibrate were selected based on our previous time course and dose-response studies (data not shown).

Analysis of mRNA expression by reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from NIT-1 cells using Trizol (Invitrogen, Carlsbad, CA) and semi-quantitative RT PCR was performed as a manufacturer guide. Primer sequences and their respective PCR fragment lengths were as follows: AdiR2 (276 bp), forward-CTTCCACACGGTGTACTGCC and reverse-AGG-GATGATTCCACTCAGGC; . PPAR-α, (330 bp) forward- CCATCTTCACGATGCTGTCC and reverse-GCTTTGGGAAGAGGAAGGTG; β-actin (353 bp), forward-GAGGCACTCTTCCAGCCTTC and re-verse-TAGAAGCATTTGCGGTGGAC.

Western blot analysis

Cells were washed in phosphate-buffered saline and extracted with general lysis buffer. Fifty microliters of the resulting protein-containing solution was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrically transferred to a nitrocellulose membrane (Amersham Bioscience, Piscataway, NJ). Each membrane was bound with pri-mary antibodies, AMPK and phosphor-AMPK (Cell Signaling, Danvers, MA) followed by rabbit secondary antibody (Santa Cruz, Santa Cruz, CA) prior to an enhanced chemiluminescence (West Zol™ ECL; iNtRON, Seoul, Korea).

Insulin secretion assay

NIT-1 cells were seeded in 1 ml of DMEM containing 25 mM glucose and 10% fetal bovine serum in a 24-well plate at 2×10^5 cells/well for 48 h. The cells were washed with HEPES-balanced KRBB containing 2 mM glucose and 0.1% fatty acid-free bovine serum albumin, and preincubated for 60 min at 37 °C in the same medium. After preincubation, the cells were stimulated with 12 mM glucose in HEPES-balanced KRBB at 37 °C for 25 min. Insulin secreted into the supernatant was measured by a radioimmunoassay (Amersham Biosciences, Piscataway, NJ) using rat insulin as standard.

Results

Regulation of AdiR2 mRNA expression by PPAR-α under hyperlipidemic conditions in NIT-1 cells

Expressions of AdiR1 and AdiR2 were analyzed in mouse insulinoma NIT-1 cells by semiquantitative RT-PCR. AdiR2 was slightly detected in control cells that had been treated with 10 μl of a solution of dimethyl sulfoxide. Only AdiR2 mRNA was elevated by palmitate after 24 h (Fig. 1). However, this elevation had decreased by 48 h (Fig. 1). The mRNA encoding PPAR-α, which is a critical factor in lipid metabolism and lipotoxicity [15], displayed the same expression pattern as AdiR2 (Fig. 1). PPAR-α agonists are synthetic ligands of PPAR-α that have been used as insulin-sensitizers for the treatment of type 2 diabetes mellitus [16]. Appropriately, we examined the role of PPAR-α in the regulation of AdiR2 under hyperlipidemic conditions using the PPAR-α agonist clofibrate. The palmitate-mediated impairment of the increase in PPAR-α mRNA expression and delay of AdiR2 and PPAR-α expression impairment by clofibrate was also confirmed (Fig. 1). PPAR-α targeted small interfering RNA (siRNA) (Fig. 2A) nearly abolished the palmitate-induced increase of AdiR2 expression in 24 h (Fig. 2B).
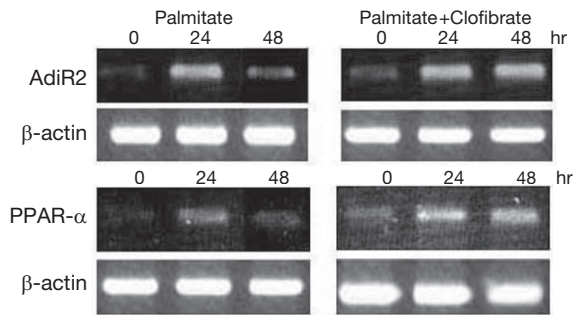
Fig. 1. Delay of AdiR2 and PPAR-α gene expression impairment by clofibrate. Semi-quantitative RT-PCR analysis of AdiR2 and PPAR-α was performed. PCR products of AdiR2 (276 bp), PPAR-α (330 bp), and β-actin (353 bp) were obtained. Each determination was independently performed three times.
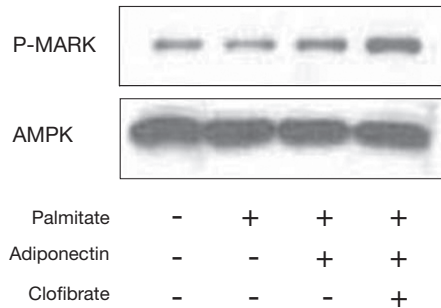

Fig. 2. Specificity of PPAR-α siRNA as determined by RT-PCR and abolishment of AdiR2 induction. At 48 h post transfection, relative PPAR-α mRNA levels were determined by semi-quantitative RT-PCR. PCR products of 330 bp and 353 bp resulted for PPAR-α and β-actin, respectively. (A) siRNA marked reduced the PCR products compared with the control group. (B) siRNA treated samples did not show evidence of marked induction compared with the control group under hyperlipidemic conditions. Each determination was independently performed three times.

Fig. 3. Induction of NIT-1 AMPK phosphorylation by full length adiponectin in the presence and absence of clofibrate. Representative Western blot analysis from protein extracts (50 μg per lane) taken from NIT-1 cells cultured 48 h under the given conditions.

Evidence of functional AdiR2 in NIT-1 cells

We confirmed a function of AdiR2 that was regulated by palmitate and PPAR-α. Adiponectin exerts most of its effects via activation of AMPK, which in turn phosphorylates and thereby inactivates acetyl CoA carboxylase. Thus, adiponectin is an AMPK activator [2, 3]. Presently, treatment of NIT-1 cells with full-length adiponectin (10μg /ml, 30 min) verified the highest phosphorylation level of AMPK in cells multi-treated with palmitate, adiponectin, and clofibrate as compared to other groups (Fig. 3).

Improvement of impaired insulin secretion via PPAR-α-induced AdiR2 expression in NIT-1 cells

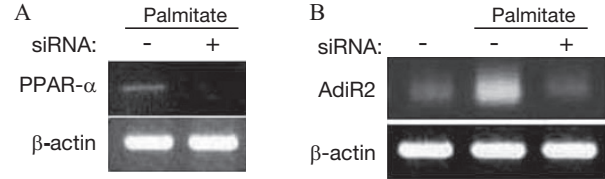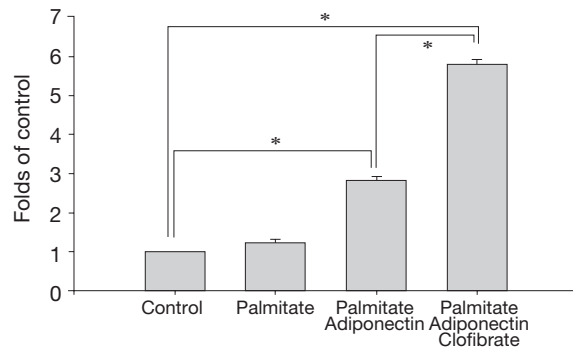Activated AMPK induces insulin secretion in pancreatic β cells [17]. We confirmed the altered insulin secretion by elevated AdiR2 expression. The insulin secretion level was higher in cells multi-treated with palmiate, adiponectin, and clofibrate, as compared with other groups except for positive control (Fig. 4), similar to AMPK phosphorylation.

Discussion

Considerable data supports a role for adiponectin in insulin action, glucose homeostasis, and possibly type 2 diabetes [18]. Furthermore, variation of the activity of the adiponectin gene is associated with type 2 diabetes in at least one study [19], although this association is not always apparent.

Adiponectin displays insulin-sensitizing effects that are mediated through at least AdiR1 and AdiR2 [20]. The present study represents the first to our knowledge to investigate the in vitro regulation of AdiR2 in response to hyperlipidemic conditions and

a PPAR-α agonist in NIT-1 cells. Similar to a previous study [21], we assessed the expressions of AdiR1 and AdiR2 by measuring their mRNA expression levels. Physiological variations in adiponectin receptor mRNA and protein levels are directly related [22]. Two related but distinct adiponectin receptors have been identified [2]; we chose to study AdiR2 because it is detectably expressed in NIT-1 cells, which are the model of the pancreatic β cells responsible for postprandial glucose uptake, and because AdiR2 appears to primarily bind the full-length adiponectin that may be the form most active in increasing fat oxidation and reducing free fatty acid levels [23].

The physiological relevance of PPAR-α in β cells is not completely understood but animal studies suggest a role of PPAR-α in pancreatic β cells under hyperlipidemic conditions [13, 24, 25]. In the present study, we attempted to clarify the mechanisms by which activation of PPAR-α ameliorates the function of β cells in type 2 diabetes in vitro model. Expression of AdiR2 mRNA in NIT-1 cells under hyperlipidemic condition was demonstrated and the regulation of AdiR2 by PPAR-α was investigated. Palmitate induced only AdiR2 mRNA expression, but this induction was abolished by PPAR-α targeted siRNA and delayed by the PPAR-α agonist clofibrate. We propose that AdiR2 may be regulated by palmitate-induced PPAR-α. However, AdiR1 mRNA expression did not exhibit a response under several conditions and AdiR2 expression was not induced by the sole application of clofibrate (data not shown). These results lead us to propose that PPAR-α may play an essential role in maintaining metabolic homeostasis via only AdiR2 in NIT-1 cells under a pathological state such as that resulting from palmitate treatment. Therefore, PPAR-α may play a different role in β cell function under pathologic and non-pathologic conditions.

It was recently reported that plasma adiponectin concentration is lowered in conditions of obesity [26]. Thus, adiponectin receptors might be acutely increased to complement impaired adiponectin effects in the liver, muscle, and adipose tissue. Acutely elevated AdiR2 in palmitate-treated cells may indicate a role of AdiR2 in mediating the beneficial effects of adiponectin in the β cell function. Interestingly, although AMPK was phosphorylated by palmitate, insulin secretion became impaired (Fig. 3, 4). In these results, it may be a phosphorylation of AMPK as an acutely inducible cell protective action [27].
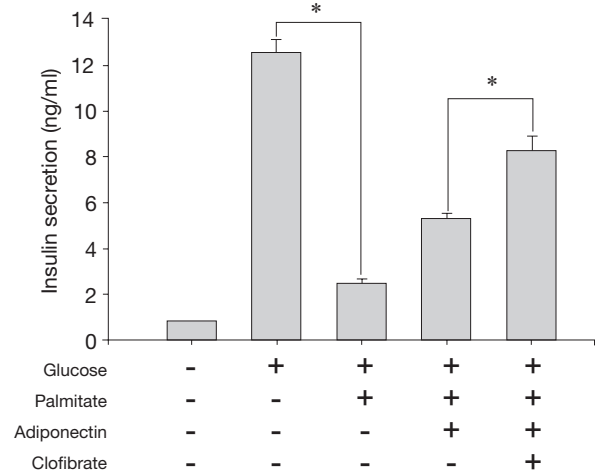

Fig. 4. Effect of static recovery with clofibrate on adiponectin-induced insulin secretion from NIT-1 cells. Insulin secretion was determined as described in the text. Statistically significant differences are indicated by *P<0.05. Each value represents the means ± standard error of the mean.

However, this induction may be too weak to improve insulin secretion. We confirmed that adiponectin spontaneously recovered impaired insulin secretion via AMPK phosphorylation (unpublished data). However, it is not yet clear whether adiponectin induces insulin secretion or not, and via AMPK or not [28, 29]. It may be dependent on kinds of insulinoma, culture conditions, etc.

In conclusion, we propose a putative mechanism by which the activation of PPAR-α can improve obesity-induced pancreatic β cell dysfunction. Firstly, activation of PPAR-α induces insulin secretion via enhancement of plasma adiponectin effects by increasing AdiR2 expression in a protective perspective against hyperlipidemic condition. Secondly, maintaining AdiR2 by PPAR-α agonist including insulin sensitizing function may also be significant in the treatment of type 2 diabetes mellitus.

References

1. T. Yamauchi, K. Hara, N. Kubota, Y. Terauchi, K. Tobe, P. Froguel, R. Nagai, T. Kadowaki, Dual roles of adiponectin/Acrp30 in vivo as an anti-diabetic and anti-atherogenic adipokine, *Curr. Drug Targets Immune. Endocr. Metabol. Disord.* 3 (2003) 243-254.

2. T. Yamauchi, J. Kamon, Y. Ito, A. Tsuchida, T. Yokomizo, S. Kita, T. Sugiyama, M. Miyagishi, K. Hara, M. Tsunoda, K. Murakami, T. Ohteki, S. Uchida, S. Takekawa, H. Waki, N. H. Tsuno, Y. Shibata, Y. Terauchi, P. Froguel, K. Tobe, S. Koyasu, K. Taira, T. Kitamura, T. Shimizu, R. Nagai, T. Kadowaki, Cloning of adiponectin receptors that mediate anti diabetic metabolic effects, *Nature* 423 (2003) 762-769.

3. B. Desvergne, W. Wahli, Peroxisome proliferator-activated receptors: nuclear control of metabolism, *Endocr. Rev.* 20 (1999) 649-688.

4. Z. Guo, Z. Xia, V. G. Yuen, J. H. McNeill, Cardiac expression of adiponectin and its receptors in streptozotocin-induced diabetic rats, *Metabolism* 56 (2007) 1363-1371.

5. N. Kubota, W. Yano, T. Kubota, T. Yamauchi, S. Itoh, H. Kumagai, H. Kozono, I. Takamoto, S. Okamoto, T. Shiuchi, R. Suzuki, H. Satoh, A. Tsuchida, M. Moroi, K. Sugi, T. Noda, H. Ebinuma, Y. Ueta, T. Kondo, E. Araki, O. Ezaki, R. Nagai, K. Tobe, Y. Terauchi, K. Ueki, Y. Minokoshi, T. Kadowaki, Adiponectin stimulates AMP-activated protein kinase in the hypothalamus and increases food intake, *Cell Metab.* 6 (2007) 55-68.

6. K. L. Mullen, A. C. Smith, K. A. Junkin, D. J. Dyck, Globular adiponectin resistance develops independently of impaired insulin-stimulated glucose transport in soleus muscle from high-fat-fed rats, *Am. J. Physiol. Endocrinol. Metab.* 293 (2007) 83-90.

7. L. C. Bollheimer, R. H. Skelly, M. W. Chester, J. D. McGarry, C. J. Rhodes, Chronic exposure to free fatty acid reduces pancreatic beta cell insulin content by increasing basal insulin secretion that is not compensated for by a corresponding increase in proinsulin biosynthesis translation, *J. Clin. Invest.* 101 (1998) 1094–1101.

8. A. B. Thrush, G. J. Heigenhauser, K. L. Mullen, D. C. Wright, D. J. Dyck, Palmitate acutely induces insulin resistance in isolated muscle from obese but not lean humans, *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 294 (2008) 1205-1212.

9. T. Coll, E. Eyre, R. Rodriguez-Calvo, X. Palomer, R. M. Sánchez, M. Merlos, J. C. Laguna, M. Vázquez-Carrera, Oleate reverses palmitate-induced insulin resistance and inflammation in skeletal muscle cells, *J. Biol. Chem.* 283 (2008) 11107-11116.

10. Oprescu, G. Bikopoulos, A. Naassan, E. M. Allister, C. Tang, E. Park, H. Uchino, G. F. Lewis, I. G. Fantus, M. Rozakis-Adcock, M. B. Wheeler, A. Giacca, Free fatty acid-induced reduction in glucose-stimulated insulin secretion: evidence for a role of oxidative stress in vitro and in vivo, *Diabetes* 56 (2007) 2927-2937.

11. F. Schuit, D. Flamez, A. De Vos, D. Pipeleers, Glucose-regulated gene expression maintaining the glucose-responsive state of beta-cells, *Diabetes* 51 (2002) S326-S332.

12. Y. T. Zhou, M. Shimabukuro, M. Y. Wang, Y. Lee, M. Higa, J. L. Milburn, C. B. Newgard, R. H. Unger, Role of peroxisome proliferator-activated receptor α in disease of pancreatic beta cells, *Proc. Natl. Acad. Sci. U. S. A.* 95 (1998) 8898-8903.

13. F. Lalloyer, B. Vandewalle, F. Percevault, G. Torpier, J. Kerr-Conte, M. Oosterveer, R. Paumelle, J. C. Fruchart, F. Kuipers, F. Pattou, C. Fiévet, B. Staels, Peroxisome proliferator-activated receptor {α} improves pancreatic adaptation to insulin resistance in obese mice and reduces lipotoxicity in human islets, *Diabetes* 55 (2006) 1605-1613.

14. E. H. Koh, M. S. Kim, J. Y. Park, Peroxisome proliferator-activated receptor (PPAR)-α activation prevents diabetes in OLETF rats: comparison with PPAR-gamma activation, *Diabetes* 52 (2003) 2331-2337.

15. T. Goto, N. Takahashi, S. Kato, K. Egawa, S. Ebisu, T. Moriyama, T. Fushiki, T. Kawada, Phytol directly activates peroxisome proliferator-activated receptor α (PPAR α) and regulates gene expression involved in lipid metabolism in PPAR α-expressing HepG2 hepatocytes, *Biochem. Biophys. Res. Commun.* 337 (2005) 440-445.

16. R. Bergeron, J. Yao, J. W. Woods, E. I. Zycband, C. Liu, Z. Li, A. Adams, J. P. Berger, B. B. Zhang, D. E. Moller, T. W. Doebber. PPAR agonism prevents the onset of type 2 diabetes in ZDF rats: a comparison to PPAR agonism, *Endocrinol.* 147 (2006) 4252-4262.

17. C. Z. Wang, Y. Wang, A. Di, M. A. Magnuson, H. Ye, M. W. Roe, D. J. Nelson, G. I. Bell, L. H. Philipson, 5-Amino-imidazole carboxamide riboside acutely potentiates glucose-stimulated insulin secretion from mouse pancreatic islets by KATP channel-dependent and -independent pathways, *Biochem. Biophys. Res. Commun.* 330 (2005) 1073-1079.

18. U. B. Pajvani, P. E. Scherer, Adiponectin: systemic contributor to insulin sensitivity, *Curr. Diab. Rep.* 3 (2003) 207-213.

19. K. Hara, P. Boutin, Y. Mori, K. Tobe, C. Dina, K. Yasuda, T. Yamauchi, S. Otabe, T. Okada, K. Eto, H. Kadowaki, R. Hagura, Y. Akanuma, Y. Yazaki, R. Nagai, M. Taniyama, K. Matsubara, M. Yoda, Y. Nakano, M. Tomita, S. Kimura, C. Ito, P. Froguel, T. Kadowaki, Genetic variation in the gene encoding adiponectin is associated with an increased risk of type 2

diabetes in the Japanese population, *Diabetes* 51 (2002) 536 -540.

20. T. Yamauchi, J. Kamon, H. Waki, Y. Terauchi, N. Kubota, K. Hara, Y. Mori, T. Ide, K. Murakami, N. Tsuboyama-Kasaoka, O. Ezaki, Y. Akanuma, O. Gavrilova, C. Vinson, M. L. Reitman, H. Kagechika, K. Shudo, M. Yoda, Y. Nakano, K. Tobe, R. Nagai, S. Kimura, M. Tomita, P. Froguel, T. Kadowaki, The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity, *Nat. Med.* 7 (2001) 941–946.

21. A. Tsuchida, T. Yamauchi, Y. Ito, Y. Hada, T. Maki, S. Takekawa, J. Kamon, M. Kobayashi, R. Suzuki, K. Hara, N. Kubota, Y. Terauchi, P. Froguel, J. Nakae, M. Kasuga, D. Accili, K. Tobe, K. Ueki, R. Nagai, T. Kadowaki, Insulin/Foxo1 pathway regulates expression levels of adiponectin receptors and adiponectin sensitivity, *J. Biol. Chem.* 279 (2004) 30817–30822.

22. K. Inukai, Y. Nakashima, M. Watanabe, N. Takata, T. Sawa, S. Kurihara, T. Awata, S. Katayama, Regulation of adiponectin receptor gene expression in diabetic mice. *Am. J. Physiol. Endocrinol. Metab.* 288 (2005) 876-882.

23. J. Fruebis, T. S. Tsao, S. Javorschi, D. Ebbets-Reed, M. R. Erickson, F. T. Yen, B. E. Bihain, H. F. Lodish, Proteolytic cleavage product of 30-kDa adipocyte complement-related protein increases fatty acid oxidation in muscle and causes weight loss in mice, *Proc. Natl. Acad. Sci. U. S. A.* 98 (2005) 2001-2010.

24. E. Tozzo, R. Ponticiello, J. Swartz, D. Farrelly, R. Zebo, G. Welzel, D. Egan, L. Kunselman, A. Peters, L. Gu, M. French, S. Chen, P. Devasthale, E. Janovitz, A. Staal, T. Harrity, R. Belder, P. T. Cheng, J. Whaley, S. Taylor, N. Hariharan, The dual peroxisome proliferator-activated receptor α/gamma activator muraglitazar prevents the natural progression of diabetes in db/db mice, *J. Pharmacol. Exp. Ther.* 321 (2007) 107-115.

25. H. Kim, M. Haluzik, Z. Asghar, D. Yau, J. W. Joseph, A. M. Fernandez, M. L. Reitman, S. Yakar, B. Stannard, L. Heron-Milhavet, M. B. Wheeler, D. LeRoith, Peroxisome proliferator-activated receptor-α agonist treatment in a transgenic model of type 2 diabetes reverses the lipotoxic state and improves glucose homeostasis, *Diabetes* 52 (2003) 1770-1778.

26. L. Qi, B. Meigs, S. Liu, J. E. Manson, C. Mantzoros, F. B. Hu, Dietary fibers and glycemic load, obesity, and plasma adiponectin levels in women with type 2 diabetes, *Diabetes Care* 29 (2006) 1501-1505.

27. J. E. Kim, M. W. Ahn, S. H. Baek, I. K. Lee, Y. W. Kim, J. Y. Kim, J. M. Dan, S. Y. Park, AMPK activator, AICAR, inhibits palmitate-induced apoptosis in osteoblast, *Bone* 43 (2008) 394-404.

28. M. Okamoto, M. Ohara-Imaizumi, N. Kubota, S. Hashimoto, K. Eto, T. Kanno, T. Kubota, M. Wakui, R. Nagai, M. Noda, S. Nagamatsu, T. Kadowaki, Adiponectin induces insulin secretion in vitro and in vivo at a low glucose concentration, *Diabetologia* 51 (2008) 827-835.

29. E. D. Targonsky, F. Dai, V. Koshkin, G. T. Karaman, A. V. Gyulkhandanyan, Y. Zhang, C. B. Chan and M. B. Wheeler, α-Lipoic acid regulates AMP-activated protein kinase and inhibits insulin secretion from beta cells, *Diabetologia* 49 (2006) 1587-1598.