The Anti-obesity Effect of *Aureobasidium pullulans* SM-2001 Extract (Polycan®) on 3T3-L1 Preadipocytes and Adipocytes

Young-Suk Kim, Jong-Min Lim, Bon-Hwa Ku, Seung-Bae Moon, Hyung-Rae Cho, Seon-Min Lee and Jung-Hee Kwon

1Glucan Co. Ltd., 25-15, Worasan-ro 950 beon-gil, Munsan-eup Jinju-si, Gyeongsangnam-do 52840, Korea
2Biological Resources Research Group, Gyeongnam Department of Environment Toxicology and Chemistry, Korea Institute of Toxicology (KIT), 17 Jeigeok-gil, Jinju 52834, Korea
3NPCChemBio Co. Ltd., Venture Support Facilities, Jinju Bioindustry Promotion Foundation, 991 Worasan-ro, Munsan-eup, Jinju-si, Gyeongsangnam-do 52839, Korea

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Obesity, the world’s leading metabolic disease, is a serious health problem in both industrialized and developing countries. Natural substances are of great interest in preventative medicine, especially in the field of metabolic syndromes— from insulin resistance to obesity and diabetes. In the present study, we investigated the effect of *A. pullulans* SM-2001 Extract (Polycan®) on the adipocyte differentiation of 3T3-L1 preadipocytes and the anti-obesity effect of 3T3-L1 adipocytes. Although β-glucan has been found to have health benefits in the regulation of the immune system and blood cholesterol levels, its role in obesity has not been fully investigated. Polycan® suppressed lipid accumulation and glycerol-3-phosphate dehydrogenase (GPDH) activity without affecting cell viability in 3T3-L1 preadipocytes and adipocytes. Polycan® also inhibited cellular lipid accumulation through down-regulation of transcription factors, such as PPARγ and C/EBPβ, and induced dose-dependent phosphorylation of AMP-activated protein kinase (AMPK)—a cellular energy sensor—while the total AMPK protein content remained unchanged. Taken together, this shows that the activation of AMPK by Polycan® in adipocytes plays a critical role in Polycan®-induced inhibition of adipocyte differentiation. Our results show that Polycan® has an anti-obesity action in vitro, suggesting a potential novel preventative agent for obesity and other metabolic diseases.

**Key words**: Adipocyte differentiation, AMPK, beta-glucan, obesity, PPARγ

**Introduction**

Obesity has become a public health crisis worldwide, and the prevalence has steadily increased over the past few decades. Recent studies estimated that obesity increases the risk of numerous of chronic diseases, such as dyslipidemia, hypertension, cardiovascular diseases, and type II diabetes [4, 34]. Adipose tissue plays a critical role in lipid metabolism and energy balance. Adipocyte differentiation, known as adipogenesis, is the anabolic process of fat cell development [30]. There is evidence that a group of closely related nuclear receptors, called peroxisome proliferator-activated receptors (PPARs), are involved in obesity. Among three PPAR isotypes identified as α, β, and γ, PPARγ is mainly expressed in adipose tissue and have been revealed to be required for the adipocyte differentiation, with the CCAAT-enhancer-binding proteins (C/EBPs) transcription factors [39]. Besides, most of the PPARγ target genes in adipose tissue are directly involved in lipidogenic pathways, including lipoprotein lipase (LPL), adipocyte fatty acid binding protein (aP2), uncoupling protein-2 (UCP-2), and glucose-transporter 4 (Glut4) [24].

Preadipocyte cell lines are useful models for investigating the adipogenesis process. 3T3-L1 preadipocyte which can be induced to differentiate into adipocyte cells, is one of the most studied preadipocyte cell lines [16, 32, 33]. During differentiation into adipocyte, PPARγ and C/EBPβ are involved in the sequential expression of adipocyte differentiation [1, 11, 26], whereas expression of PPARα and C/EBP, which trigger the expression of adipocyte-specific proteins, are induced during terminal differentiation of the adipocyte lineage [14, 29].

Many natural extracts have drawn attention because of
their health benefits as well as their relative safeness. An accumulated evidence indicates that natural extracts shows physiological properties in anti-obesity and anti-diabetic effects [3]. Red yeast rice extracts suppress adipogenesis in 3T3-L1 model [19], and pine needle extract suppresses differentiation of 3T3-L1 and obesity in rats receiving high-fat diets [18].

β-Glucan is a fibre-type complex sugar (polysaccharide) derived from the cell wall of baker’s yeast, oat and barley fibre, and many medicinal mushrooms. The two primary uses of β-glucan are enhancement of the immune system [7, 12] and lowering of blood cholesterol levels [2, 31]. In addition, some reports revealed the hypoglycaemic effect of β-glucan extracts from plants or mushroom in animal experiments [9, 27] and clinical trials [37], whereas β-glucan derived from other origins showed no hypoglycaemic effects on the STZ-induced diabetes [5, 40, 41]. However, Polycan® extracted from a UV-induced mutant of A. pullulans (SM-2001), mainly containing β-1,3/1,6-glucan [18] showed relatively favourable effects against diabetic complications, particularly diabetic nephropathy and hepatopathy [41], and abnormalities in lipid metabolism [5]. Versatile roles of β-glu- cans have to be further investigated in obesity and other metabolic syndrome.

In the present study, we explored the effect of Polycan® on adipocyte differentiation in 3T3-L1 preadipocyte model to gain further insight into the role of β-glucan in adipogenesis and obesity.

Materials and Methods

Chemicals and materials

Polycan® were supplied by Glucan Corp. (Korea) and were stored in a refrigerator at 4°C to protect from light and degradation. 3T3-L1 cells were purchased from the American Type Culture Collection (Manassas, VA, U.S.A.). Dulbecco’s Modified Eagle Medium/Ham’s F-12 nutrient mixture (DMEM/F12), fetal bovine serum (FBS), calf bovine serum (CBS), penicillin-streptomycin, phosphate buffered saline (PBS) and transferrin were obtained from Gibco BRL (Rockville, MD, U.S.A.). Dexamethasone (DEX), insulin, 1-methyl3-isobutylxanthine (IBMX), GPDH assay kit and Monoclonal Anti-β-actin were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). C/EBPα (D56F10) XP® Rabbit mAb and PPARγ (81B8) Rabbit mAb were purchased from Cell Signaling Technology (Danvers, MA, U.S.A.). The lip-olyssis assay kit was purchased from AMS Biotechnology (Abingdon, Ox., U.K.). Ethanol and other chemicals were analytical reagent grade.

Preparation of A. pullulans SM-2001 extract (Polycan®)

We used a A. pullulans SM-2001 extract, Polycan® (Glucan Co., Ltd., Korea) [40]. Liquid culture of A. pullulans SM-2001 was extracted at 121°C for 15 min, and then filtered (1 um pore size) for 2 times. The filtrate was sterilized (80°C, 30 min) and freeze dried. The Polycan® used in the experiment was used by dissolving by concentration through distilled water. The contents of glucan were determined by kit β-Glucan Assay Kit (Megazyme, Chicago, USA). In Polycan®, around 90% of the total glucan consists of β-1,3/1,6-glu- cans and the rest is α-glucan (pullulan).

3T3-L1 cell culture and cell viability assay

Mouse embryo preadipocyte (3T3-L1) cell lines was obtained from ATCC (American Type Culture Collection, Manassas, VA, U.S.A.) and cultured in Dulbeco’s modified Eagle’s medium (DMEM) containing 10% calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin in a humidified atmosphere of 5% CO2 at 37°C. Cell viability was measured using the CCK-8 kit (Dojindo, Kumamoto, Japan), according to the manufacturer’s instructions. Briefly, the cells were plated in 96-well plates at a density of 1×10⁵ cells/well. After overnight incubation, the cells were treated with the 0, 100, 200, 400 µg/ml Polycan® to be tested (at the concentration as indicated) and cultured for 24 hr. After incubation, the CCK-8 solution was added to each well and incubated for 3 hr at 37°C. The absorbance at 450 nm was measured using a microplate reader (Bio-Tek Company, Winooski, VT, U.S.A.). The experiments were performed in triplicate.

3T3-L1 adipocyte differentiation and Oil Red O staining

Adipocyte differentiation was induced in 100% confluent 3T3-L1 cells with adipogenic medium (DMEM containing 10% CBS with 0.5 mM IBMX, 1 µM dexamethasone, and 1 µg/ml insulin) treated with different concentration of Polycan® (0, 100, 200, 400 µg/ml). Two days after induction, the medium was changed to DMEM including 10% FBS and 1 µg/ml insulin with the samples for additional two days. The cells were then maintained in DMEM with 10% FBS for another four days. Lipid accumulation in adipocytes was estimated by staining with Oil Red O. Six days after the ini-
Fig. 1. Experimental scheme. For the induction of adipocyte differentiation, 3T3-L1 preadipocytes were seeded. At confluence (day 0), the cultured preadipocytes were induced to differentiate by the addition of differentiating medium containing 0.5 mM methylisobutylxanthine (IBMX), 1 μg/ml insulin, and 1 μM dexamethasone (Dex) from day 0 to day 2. At day 2, medium was changed with medium containing 1 μg/ml insulin for an additional 2 days from day 2 to day 6. The medium was refreshed every 2 days. At day 6, differentiated 3T3-L1 cells were subjected to Oil red O solution or used for Western blot analysis. Polycan® was added to the cell culture medium at concentrations of 0, 100, 200 and 400 μg/ml from day 0 to day 6. DMEM, Dulbecco’s modified Eagle’s medium.

Lipolysis assay

Amounts of glycerol released from cells into the medium were measured to analyze the lipolytic effect of Polycan® on the triacylglycerol accumulated in adipocytes. Medium was collected from the culture plate and heated at 65°C for 8 min to inactivate enzymes released from the cells. The glycerol was measured with a commercial lipolysis assay kit (AMS biotechnology, Abingdon, OX., U.K.). Cellular protein content was analyzed with a BCA protein assay kit (Pierce, Rockford, IL., U.S.A.) using BSA as a standard.

GPDH activity

The 3T3-L1 adipocytes were harvested 48 hr after initiation of differentiation or 6 days after differentiation with 0, 100, 200, 400 μg/ml Polycan®. Cells were carefully washed twice with ice-cold PBS and collected with a scraper into 300 μl of 100 mM triethanolamine/HCl buffer, pH 7.5, 2.5 mM EDTA. The harvested cells were sonicated in ice at 25 ultrasonic burst of 10 sec each in a DU-250 Bioruptor with a maximum output power of 250 W (Tosho Denki, Co. Ltd., Japan). After centrifugation at 13,000× g for 5 min at 4°C, the supernatants were assayed for GPDH activity according to the method of Wise and Green [17]. GPDH activity was measured under zero-order kinetics and optimal substrate and cofactor conditions at 25°C for 180 sec in a spectrophotometer (Beckman Coulter, DU 530, Indianapolis, IN, U.S.A.). The standard reaction mixture contained 100 mM triethanolamine/HCl buffer (pH 7.5), 2.5 mM EDTA, 0.1 mM/2-mercaptoethanol, and 0.12 mM NADH. The reaction was initiated by the addition of 0.2 mM dihydroxyacetone phosphate, and the rate of NADH oxidation was measured by a change in absorbance at 340 nm for 60 sec. Enzyme activity (%) was expressed as percent against control (100%) [18].

Western blot analysis

For Western blot analysis, cells (3×10⁶) were cultured in 3 ml of DMEM and differentiated to the adipocytes by incubating in DMEM containing 10% CBS, 0.5 mM IBMX, 1 μM dexamethasone, and 1 μg/ml insulin. The cells were harvested 48 hours after initiation of differentiation or 6 days after differentiation with 0, 100, 200, 400 μg/ml Polycan®, washed twice in PBS and then dissolved for 30 min with lysis buffer [150 mM NaCl, 50 mM Tris (pH 7.2), 1 mM EDTA, 0.5% sodium deoxycholate, 1% Nonidet P-40, 1 mM sodium vanadate, 1 mM NaF, 20 μg/ml aprotinin, 50 μg/ml...
leupeptin, 10 μg/ml pepstatin A and 100 μg/ml phenylmethylsulfonyl fluoride. Finally, the solution was centrifuged at 14,000 × g for 20 min at 4 °C. The supernatant was collected and protein concentrations were determined by the Bradford method. Proteins (30 μg) were loaded onto each lane for 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the separated proteins were blotted to PVDF membrane. The membrane was blocked with 5% non-fat milk in PBST buffer for 1 hr at room temperature. Then, the membrane was washed three times with PBST followed by incubation with secondary antibodies for 1 hr at room temperature. Bands were visualized using ECL solution (Thermo Scientific) and quantified using the Chemidoc Imaging System (Bio-Rad; Hercules, CA, U.S.A.).

Statistical analysis
The results were analyzed using Prism version 5.00 software (GraphPad Software, San Diego, CA, U.S.A.). One-way ANOVA was applied to calculate the significance between the groups. Statistical significance was indicated by a p value of <0.05. Data were expressed as the mean ± SEM of three independent experiments.

Results and Discussion

Proliferation of preadipocytes
To identify whether Polycan® (A. pullulans SM-2001 Extract) inhibited the proliferation of 3T3-L1 cells, preadipocyte were treated with Polycan® at the concentration of 0, 100, 200, or 400 μg/ml. As shown in Fig. 2, cell viability was not affected by treatment of Polycan® at all concentrations in 3T3-L1 preadipocyte, showing that Polycan® are not cytotoxic in 3T3-L1 preadipocytes. This result is consistent with a previous study reporting that β-glucan-rich polysaccharide was not cytotoxic toward 3T3-L1 cells [21]. Although a few investigators have reported β-glucan-mediated down-modulation [35, 42], most of the research to date describes a positive modulatory role. Similarly, exopolymers derived from A. pullulans SM-2001, an ultraviolet (UV)-induced mutant strain, also contain -1,3/1,6-glucans showing various pharmacological activities [40] and β-Glucan is a component of fungal cell walls that modulates many processes in vivo and in vitro [12].

Effect of Polycan® on lipid accumulation
The effect of Polycan® on intracellular lipid accumulation was examined by oil red O staining (Fig. 3A). To test the inhibitory effects of Polycan® on lipid accumulation in 3T3-L1 cells, insulin, DEX and IBMX were used to induce 3T3-L1 pre-adipocytes to differentiation. At the concentration of 0, 100, 200 and 400 μg/ml Polycan® inhibited the adipocyte differentiation (Fig. 3B). Quantitative data was obtained through extraction of Oil-Red O-stained cells with isopropanol and spectrophotometric analysis. The O.D. absorbance significantly decreased (p<0.05) in cells treated with Polycan® in dose-dependent manner (Fig. 3B), and the decrease rates were 51.2%, 36.03%, and 18.3% at concentrations of 100, 200 and 400 μg/ml of Polycan®, respectively. These results indicated that Polycan® could inhibit adipocyte differentiation, thus inhibit intracellular lipid accumulation in 3T3-L1 cells.

Effects of Polycan® on glycerol release
To clarify the direct effect of Polycan® on lipolysis, the amount of glycerol released into the medium was measured (Fig. 4). The amount of glycerol in the medium was increased by 78% in the presence of 400 μg/ml Polycan®. In the present study, it was clear that Polycan® treatment decreased the intracellular lipid content in 3T3-L1 adipocytes (Fig. 3) as well as increased the amount of glycerol released into the medium, indicating activation of lipolysis.

Effect of Polycan® on GPDH activity
To ascertain the inhibition of the accumulation of intracellular lipid in 3T3-L1 preadipocytes, we examined the effect of Polycan® on GPDH activity. GPDH is an index of differentiation as one of the lipid-synthesizing enzymes expressed in adipocytes differentiated from preadipocytes.
Fig. 3. Effects of Polycan® on intracellular lipid accumulation in 3T3-L1 cells. 3T3-L1 cells were treated with polycan (0, 100, 200, 400 μg/ml) for day 6. (A) The mature adipocytes were stained with Oil-red O, and the (B) OD value were measured to quantify intracellular lipid content. Three independent experiments were performed and the data were shown as mean ± SD. Values do not share the same letter are significantly different (p<0.05). ***p<0.001.

Fig. 4. Effects of Polycan® on glycerol release in 3T3-L1 adipocytes. The differentiated 3T3-L1 adipocytes were treated in serum-free medium with Polycan® (0, 100, 200, 400 μg/ml). The medium was collected and assayed for glycerol content. Three independent experiments were performed and the data were shown as mean ± SD. Values do not share the same letter are significantly different (p<0.05). ***p< 0.001, **p<0.01, *p<0.05.

Fig. 5. Effect of Polycan® on GPDH activity in cultured 3T3-L1 adipocytes. The 3T3-L1 adipocytes were harvested 6 days after the initiation of differentiation with 0, 100, 200, 400 μg/ml Polycan®. Three independent experiments were performed and the data were shown as mean ± SD. Values do not share the same letter are significantly different (p<0.05). *** p<0.001.

Cultured 3T3-L1 preadipocytes and adipocytes were exposed to Polycan® at various concentrations, and then the cells were differentiated with a differentiation medium or DMEM after differentiation. As shown in Fig. 5, the treatment of 3T3-L1 preadipocytes and adipocytes with Polycan® significantly inhibited GPDH activity dose dependently. This result demonstrates for the first time, to the best of our knowledge, Polycan® causes a significant decrease in the activity of GPDH in 3T3-L1 preadipocytes without eliciting cell cytotoxicity, suggesting that Polycan® may block adipogenesis, at least in part, by down-regulating key adipogenic transcription factors in 3T3-L1 preadipocytes and may have antiatherogenic, anti-inflammatory, and antidiabetic effects through down-regulation of GPDH in 3T3-L1 adipocytes.

Effect of Polycan® on expression and activity of adipogenic proteins

Above results demonstrated that Polycan® inhibits intra-
cellular lipid accumulation and GPDH activity without toxicity to 3T3-L1 adipocytes. Therefore, we examined the effect of Polycan® on the expression of adipogenic factors in 3T3-L1 adipocytes. Adipocyte differentiation involves a series of programmed changes in adipogenic protein expression. To determine whether reduced lipid accumulation and GPDH activity resulted from Polycan®-mediated alteration in the differentiation program, we examined the expressions of adipogenic proteins by Western blot analysis. As shown in Fig. 6, treatment with Polycan® reduced the protein levels of major adipogenic transcription factors, PPARγ, C/EBPα and pAMPK in 3T3-L1 preadipocytes. In 3T3-L1 adipocytes, treatment with Polycan® also dose-dependently decreased the protein levels of PPARγ, C/EBPα whereas increased the phosphorylation of AMPK (Fig. 6). PPARγ expression was significantly decreased concomitantly with reduction of C/EBPα protein expression in 400 μg/ml polycan group compared to the control group (Fig. 6). Polycan® effectively inhibited the differentiation medium-induced increase of PPARγ expression in 3T3-L1 adipocytes at concentrations greater than 100 μg/ml. Adipogenesis is the process by which precursor stem cell differentiate into lipid laden adipocytes adipocytes [15]. This process is regulated by transcriptional activators such as PPARγ and C/EBPα [6, 33]. These transcription factors were known to regulate the middle and late stages of adipocyte differentiation [20]. Also, FABP4 is a differentiated adipocyte marker gene that is transcriptionally regulated by PPARγ [43, 44]. Although C/EBPα is an important factor in terminal differentiation of adipocytes, knockout of C/EBPα in adipocytes did not show insulin sensitivity [10, 38]. It means that C/EBPα is an essential factor in which adipocyte acquires insulin sensitivity. As obesity and insulin resistance are strongly linked to the accumulation of excessive lipids [13], maintenance of C/EBPα by treatment of Polycan® has significant meaning in improving insulin resistance accompanying excess lipid accumulation. PPARγ is capable of promoting adipogenesis in C/EBPα-deficient cells, whereas C/EBPα is incapable of promoting adipogenesis in PPARγ-deficient cells. These findings demonstrate that PPARγ is a more important master regulator of adipogenesis than C/EBPα [38]. The effect of Polycan® on these factors was specific since the levels of β-actin were unaffected. In adipose tissue, lipolysis activates AMP-activated protein kinase (AMPK). AMPK acts as a fuel sensor and regulates glucose and lipid homeostasis in adipocytes [28]. Once activated, AMPK phosphorylates a number of proteins and modulates the transcription of genes implicated in the regulation of energy metabolism to switch on catabolic pathways that produce ATP and switch off anabolic pathways that consume ATP [8].

Several studies already have reported that β-glucan inhibits adipocyte differentiation and improves serum lipid levels in high fat diet-induced obese rat models [22, 25]. However, they all used β-1,3-glucan, not β-1,3/1,6-glucan evaluated in the present study. Although both β-1,3-glucan and β-1,3/1,6-glucan have suppressive function in obesity, the latter is more structurally stable compared to β-1,3-glucan because of its innate glycosidic bond. Therefore, β-1,3/1,6-glucan has merits in higher yields in the production process as anti-obesity materials. To the best of our knowledge, the results of the present study demonstrate, for the first time, that Polycan® consisting mainly of β-1,3/1,6-glucan can have anti-obesity effects in 3T3-L1 adipocyte.

The nuclear receptor PPARγ and members of the C/EBPα complex synergistically activate downstream promoters of adipocyte-specific genes, such as acetyl-CoA carboxylase, acyl CoA synthase and GPDH. Polycan® inhibits cellular lipid accumulation through down-regulation of transcription factors such as PPARγ and C/EBPα and up-regulation of phosphorylated AMPK. Consistent with our data showing that the expression of cytosolic GPDH was enhanced by high levels of PPARγ in adipocyte [36], there were reduced activity of GPDH and decreased level of PPARγ in Polycan® treated adipocyte.

In conclusion, we report that Polycan® exerts an anti-obesity effect through inhibition of the expression of key transcription factors and genes responsible for adipocyte differentiation. In addition, we have shown that activation of...
AMPK by Polycan® in adipocytes plays a critical role in Polycan®-induced inhibition of adipocyte differentiation. Taken together, Polycan® may have novel preventative potential for obesity and other metabolic diseases, warranting further investigation for the precise mechanism.

The Conflict of Interest Statement

The authors declare that they have no conflicts of interest with the contents of this article.

References


초록: 3T3-L1세포에서 흑효모 SM-2001 추출물(Polycan®)의 항비만 효과

김영숙1, 임종민1, 구본화1, 문승배1, 조형래1, 이선민2, 권정희3*
(1(주)글루칸, 2안전성평가연구소 생명자원연구그룹, 3(주)엔피켐바이오)

비만은 이제 선진국 뿐만 아니라 개발도상국에서도 가장 문제가 되는 대사성 질환이다. 최근 치료보다는 예방에 중점을 두는 예방의학 시대가 도래함에 따라, 인슐린 저항성 비만과 당뇨병 등의 대사 증후군을 예방하기 위한 천연물들이 큰 관심을 받고 있다. 특히, 베타글루칸은 면역계와 혈중 콜레스테롤 조절에 이로운 효과가 있는 것으로 알려져 있지만 비만에 미치는 영향에 대해서는 완전히 밝혀지지 않았다. 이에 본 연구에서는 β-1,3/1,6-glucan의 함량이 전체 글루칸 함량의 90% 이상 되는 자체 개발 흑효모 균주 SM-2001 추출물(폴리칸)이 3T3-L1 지방전구세포의 지방세포화에 미치는 영향을 조사하였다. 폴리칸은 지방전구세포에서 지방축적과 GPDH 효소 활성을 억제하는 것으로 나타났다. 이는 폴리칸이 세포 내에서 지방의 축적을 조절하는 전사인자인 PPARγ와 C/EBPα의 하향조절과 세포 내 에너지 센서 역할을 하는 AMPK 효소의 인산화를 유도함으로써 항비만 효과를 발현하는 것으로 확인되었다. 본 연구를 통해 폴리칸의 항비만 효과를 확인하였으며, 향후 비만과 대사성 질환을 예방할 수 있는 식의약 소재로서 그 활용가치를 더욱 높일 수 있을 것으로 기대한다.