Endocr (2010) 37:274–279
DOI 10.1007/s12020-009-9298-4

ORIGINAL ARTICLE

Proteasome activity correlates with male BMI and contributes to the differentiation of adipocyte in hADSC

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Received: 17 September 2009 / Accepted: 16 December 2009 / Published online: 5 January 2010

Abstract We have previously reported that 26S proteasome subunit mRNA expressions correlate with male body mass index (BMI). In this study, to investigate whether proteasome activities are correlated with BMI, we recruited 61 healthy young Japanese male subjects, measured proteasome activities in their plasma, and correlated them with their BMI and various metabolic factors. We found that among three different proteasome activities, chymotrypsin-like activity in plasma was positively correlated with BMI in healthy Japanese male subjects. Furthermore, we analyzed proteasome activity in vitro during the differentiation of human adipose-derived stem cell (hADSC) into mature adipocytes. In the early stage of differentiation, proteasome activity was at its highest level, and proteasome inhibitor could inhibit hADSC adipocyte differentiation. Our findings suggest that proteasome is an important controlling factor for the development of obesity and adipogenesis.

Keywords Proteasome activities · Obesity · BMI · Adipogenesis · hADSC

Introduction

Obesity has become a major public health problem and is a causative factor of many chronic diseases and adverse health consequences, including diabetes mellitus type 2 [1], dyslipidemia [2], hypertension [3], and coronary artery disease [4]. Obesity develops not only from the imbalance of caloric intake and energy expenditure but also environmental, (e.g., physical activity) and inherited factors [5], (e.g., single nucleotide polymorphism (SNP) of the obesity-associated gene (FTO) [6]. Many researchers have investigated the genetics of obesity and many obesity-related factors, e.g., leptin [7], uncoupling proteins [8, 9], peroxisome proliferator-activated receptor γ (PPARγ) [10], adiponectin [11], and others have been discovered; however, obesity develops from adipocyte differentiation and subsequent fat accumulation [12–14].

In a recent study, we showed that 26S proteasome subunit mRNA expressions correlate with male obesity [15]. The 26S proteasome has multi-subunit protease complexes consisting of 20S proteasome harboring proteolytic active sites and 19S caps that execute regulatory functions [16]. Proteasome is the main proteolytic enzyme that functions in the ubiquitin–proteasome system, regulation of the cell cycle [17], and apoptosis [18]. The ubiquitin–proteasome system also influences the development of obesity, and insulin signaling and secretion in type 2 diabetes. Briefly, the lipid synthesis in adipocytes and lipid production by the liver is regulated through the ubiquitin–proteasome system. In insulin signaling, the binding of insulin to insulin receptor induces phosphorylation of the insulin receptor substrate (IRS) and leads to activation of multiple signaling pathways. Phosphorylated IRS proteins are inactivated by degradation through the ubiquitin–proteasome system [19]. However, the relationship between proteasome activities and BMI is still unknown.

In this study, we tested whether proteasome activities in plasma may correlate with BMI in randomly selected

Electronic supplementary material The online version of this article (doi:10.1007/s12020-009-9298-4) contains supplementary material, which is available to authorized users.

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Japanese males from Tokushima University (Tokushima, Japan). Furthermore, we analyzed proteasome activity during preadipocyte differentiation, using hADSC, which can differentiate into various lineages [20, 21].

Results

Proteasome activities in plasma

To identify whether plasma proteasome activities correlate with BMI and various metabolic factors, we recruited young Japanese subjects who were confirmed to be healthy with no apparent medical problems (Table 1). Eukaryotic proteasome have three different active sites: chymotrypsin-like, trypsin-like, and caspase-like. By measuring three different proteasome activities in plasma, only chymotrypsin-like activity was positively correlated with BMI ($r = 0.343$, $P = 0.007$), body fat ($r = 0.302$, $P = 0.018$), total cholesterol ($r = 0.337$, $P = 0.008$) and triglyceride ($r = 0.313$, $P = 0.014$) in male subjects (Fig. 1).

Proteasome activities during adipocyte differentiation in hADSC

Obesity is associated with environmental and inherited factors; however, in all cases it develops by fat accumulation; therefore, we assumed that proteasome activity contributes to adipocyte differentiation. We then analyzed proteasome activities during hADSC differentiation into mature adipocytes. On the sixthday after induction of differentiation, all proteasome activities were highest, especially trypsin-like and caspase-like activities, which were significantly increased to about 4- and 2.5-fold, respectively (Fig. 2). After the sixth day, all proteasome activities decreased.

Effect of proteasome inhibitor on hADSC differentiation

We found that proteasome activity was highest in the early stage of adipocyte differentiation; therefore, we predicted that inhibition of proteasome activity might reduce adipocyte differentiation. To investigate this assumption, we analyzed the effect of proteasome inhibitor carbobenzoxyl-leucinylleucinyl-leucinal (MG132) on adipocyte differentiation in hADSC. MG132 is one of the most widely used proteasome inhibitors. This agent is a substrate analogue and a highly potent transition-state inhibitor (*Ki* of MG132 is a few nM of chymotryptic activity of pure 20S proteasomes, and its IC_{50} is a few μM for the inhibition of proteolysis in cultured cells) [22, 23]. Various doses of MG132 (1, 10, 100 nM) were added to cell cultures during adipocyte differentiation, and on the 6th, 12th, and 18th day after induction of differentiation, cells were stained with Oil Red O to examine lipid accumulation. As a result, MG132 was found to inhibit hADSC lipid accumulation in a dose-dependent manner (Fig. 3). After the 18th day from induction, removing MG132 from the cells accumulated lipid droplets as in the control. This concentration of MG132 did not affect the cell viability (see Supplementary Fig. S1) and at 100 nM MG132, proteasome activity was inhibited (see Supplementary Fig. S2).

Next, to investigate whether MG132 affects the expression of dipocyte-specific transcription factors PPARγ and C/EBPβ, we performed Western blot analysis. The expressions of PPARγ and C/EBPβ were significantly reduced in hADSC cells treated with a dose of 100 nM MG132 (Fig. 4). Hence, the proteasome inhibitor MG132 could inhibit hADSC adipocyte differentiation.

Table 1 Characteristics of the recruited 61 male subjects

Age (y)	23.7 ± 0.4
BMI (kg/m^2)	22.3 ± 0.4
Total body fat (%)	18.9 ± 0.7
Total cholesterol (mg/dl)	172.7 ± 3.6
HDL cholesterol (mg/dl)	56.7 ± 1.4
Triglyceride (mg/dl)	78.0 ± 5.2

Mean ± S.E.M

Discussion

In a recent study, we demonstrated that the expression levels of many proteasome subunits mRNA had significant negative correlations with male BMI [15]. In a recent study, Chang et al. [24] reported similar results after studying their 31 volunteers and finding that plasma ubiquitin was significantly decreased in obese individuals versus normal controls, and its levels were found to be inversely correlated with individuals' BMI. In this study, we showed that proteasome activity has significant positive correlations with BMI, body fat, serum total cholesterol, and triglycerides in male subjects. This opposite result might be ascribed to an auto-regulatory feedback mechanism. Several 26S proteasome subunit mRNAs knocked-down by double-stranded RNA interference (RNAi) significantly decreased mRNA and protein levels of targeted subunits of 20S proteasome, but promoted the high expression of many non-targeted subunits [25]. Moreover, treatment of COS7, Hek293, HeLa, and T2 cells with proteasome inhibitors induced transient and concerted up-regulation of all mammalian 26S proteasome subunit mRNAs [26]. From these findings, it is suggested that the increase of

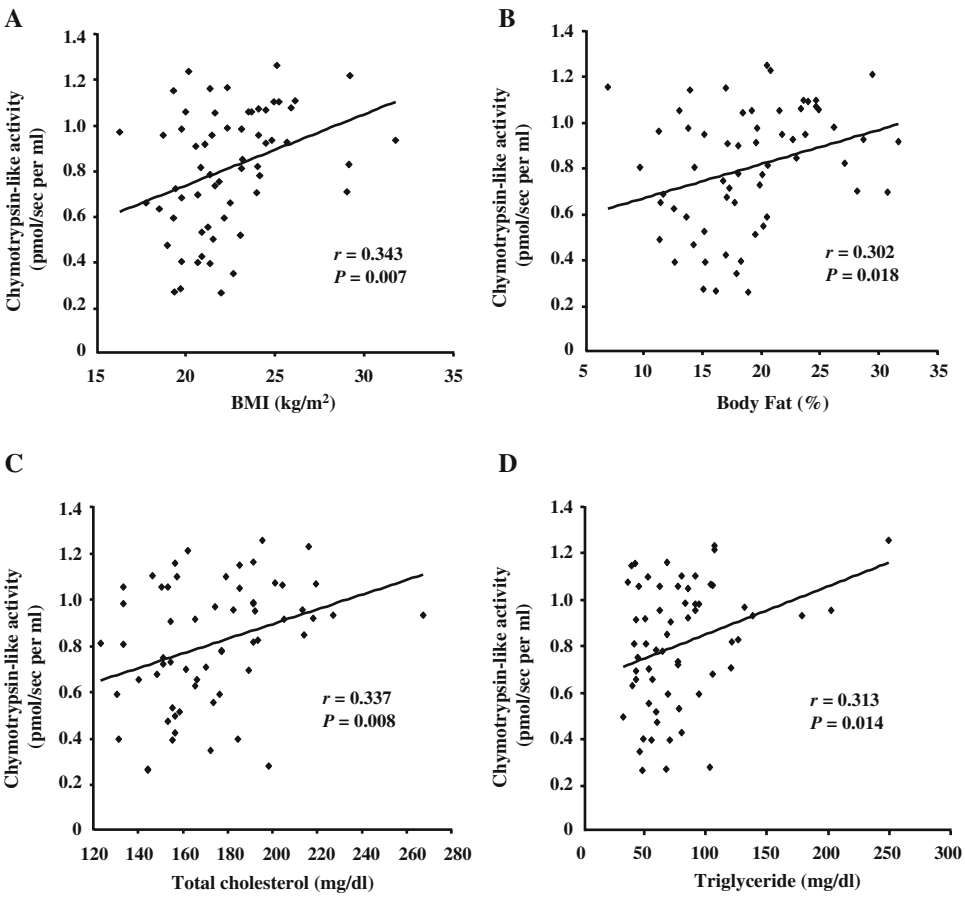

Fig. 1 Plasma proteasome activity. Correlation between proteasome (chymotrypsin-like) activity and **a** BMI (Pearson correlation, $r = 0.343$, $P = 0.007$, $n = 61$), **b** body fat ($r = 0.302$, $P = 0.018$, $n = 61$), **c** total cholesterol ($r = 0.337$, $P = 0.008$, $n = 61$), and **d** triglyceride ($r = 0.313$, $P = 0.014$, $n = 61$)

proteasome activity in plasma down-regulates proteasome subunit mRNA expression.

Among three different proteasome activities, only chymotrypsin-like activity was positively correlated with BMI, body fat, total cholesterol, and triglyceride. Chymotrypsin-like activity is considered the most important element for protein breakdown, and the only activity that has to be assayed to assess the capacity of proteasome to degrade proteins [27]. Hence, it is quite reasonable to find that chymotrypsin-like activity was the only activity of proteasome to be correlated with BMI and other obesity-related factors.

Next, we analyzed proteasome activity in vitro during adipocyte differentiation in a cell line, hADSC. The results showed that proteasome activities were highest in the early stages of adipocyte differentiation. Adding a proteasome inhibitor, MG132, inhibited the cellular differentiation process into mature adipocytes, and decreased the expressions of PPARγ and C/EBPβ, known as master regulators of adipogenesis. Furthermore, the release of MG132 re-induced proadipocyte differentiation into mature cells. In a previous study that described 3T3-L1 adipocytes, C/EBPβ was immediately expressed after the induction of differentiation; growth-arrested 3T3-L1 preadipocytes synchronously re-entered the cell cycle and underwent several rounds of mitotic clonal expansion. Subsequently, PPARγ and C/EBPα were expressed approximately on the sixth day post-initiation of differentiation and 3T3-L1 cells differentiated into mature adipocyte [28, 29]. In hADSC little is known about the progression of the differentiation program, but hADSC needs long time to accumulate lipid compared with 3T3-L1 cells. And our data showed that MG132 decreased PPARγ and C/EBPβ expression. These findings suggest that around sixth day after induction of differentiation, proteasome activities are at its highest level, just at this time the cells undergo mitotic clonal expansion, and MG132 inhibit the differentiation.

In conclusion, we report here that proteasome activity has significant positive correlations with male BMI, body fat, serum total cholesterol, and triglyceride. Furthermore, in early stage of hADSC differentiation into mature adipocytes, proteasome activity was at its highest level, and proteasome inhibitor could inhibit adipocyte differentiation. We suggest that men with high BMI usually have high rates of adipocyte differentiation as well as high levels of plasma proteasome activities. Proteasomes may comprise important controlling factors for the development of obesity.

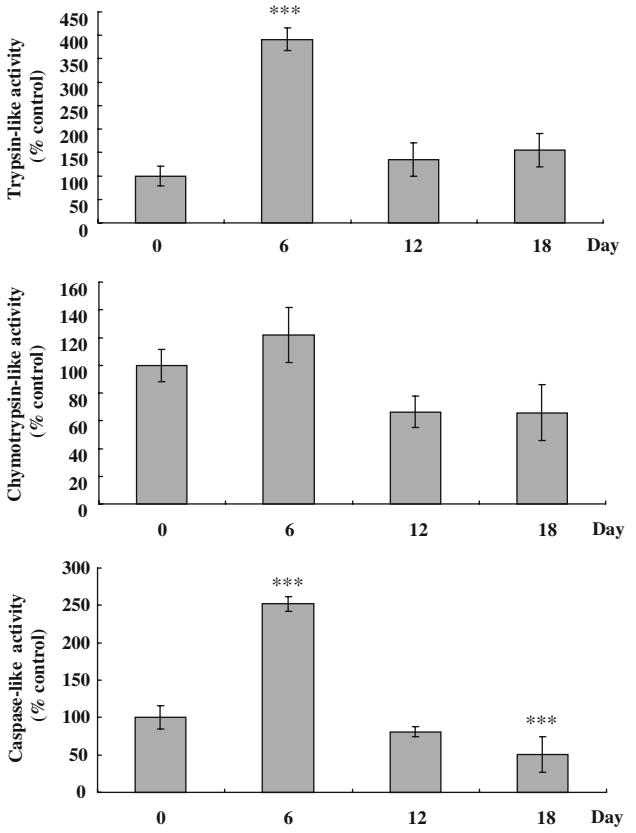

Fig. 2 Proteasome activity during hADSC adipocyte differentiation. Trypsin-like, chymotrypsin-like, and caspase-like activities were measured 0, 6, 12, and 18 days after induction of differentiation. Data are the means of four samples ± SD as a percentage from 0 day. *** $P < 0.001$ significantly different from 0 day by one-way ANOVA; Bonferroni's multiple comparison test

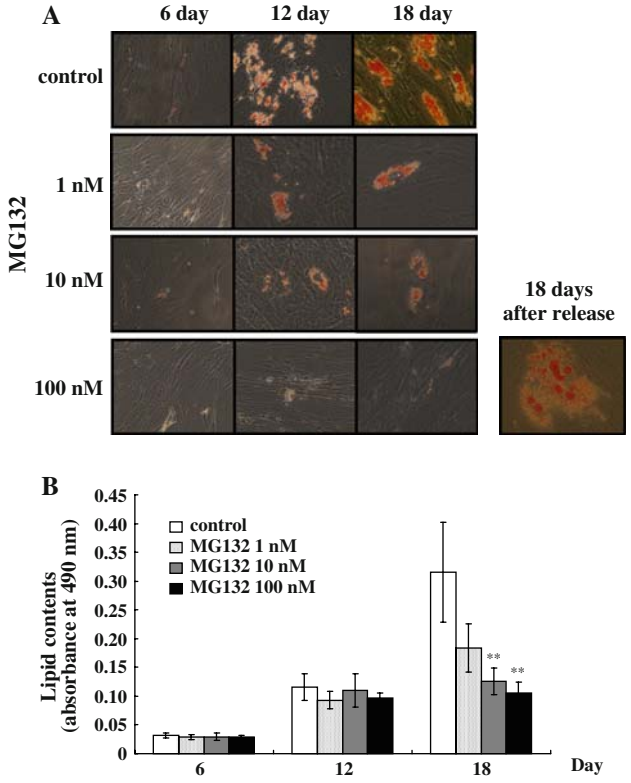

Fig. 3 Effect of MG132 on lipid accumulation in hADSC. hADSC differentiation into adipocytes was induced as described in "Materials and methods" section with MG132 at various concentrations (0, 10, 100 nM) for 18 days. **a** On the 6th, 12th, 18th day after induction, and on the 18th day after release from 18th day treatment of 100 nM MG132, cells were stained with Oil Red O. **b** Stained oil droplets were dissolved in ethanol and quantified by spectrophotometric analysis at 490 nm. Data are the means of six samples ± SD. ** $P < 0.01$ significantly different from control by one-way ANOVA; Bonferroni's multiple comparison test

Materials and methods

Subjects

Sixty-one young male Japanese students from the University of Tokushima (Tokushima, Japan) participated in this study. Prior to their inclusion they were confirmed to be generally healthy with no apparent medical problems. Informed consent was obtained from all participants, and the study was approved by the medical ethics committee of the University of Tokushima, Tokushima, Japan. BMI and other metabolic factors were then measured, as previously described [9] and body fat was measured by Body Fat Analyzer TBF-310 (Tanita Corp., Tokyo, Japan). After overnight fasting, 10 ml of venous blood were taken from each participant under sterile conditions, following centrifugation; plasma samples were obtained and stored at −80°C until analysis. Serum total cholesterol, high density lipoprotein (HDL), and triglyceride were automatically measured using Automatic analyzer 7150 (Hitachi High-Technologies Corp., Tokyo, Japan).

Proteasome activity assay

Chymotrypsin-like, trypsin-like, and caspase-like activities of the proteasome in plasma and cell lysates were assayed by hydrolysis of the fluorogenic peptides succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin 4-amino-7-methylcoumarin (SUC-LLVY-AMC) (Sigma, St. Louis, MO, USA), butyl-oxycarbonyl-Leu-Ser-Thr-Arg-7-amido-4-methylcoumarin (BOC-LSTR-AMC) (Sigma), and acetyl-L-norleucyl-L-prolyl-L-norleucyl-L-aspartyl-methylcoumarylamide (Ac-nLPnLD-AMC) (BIOMOL International LP, Plymouth Meeting, PA, USA), respectively, as described previously [27]. Aliquots of plasma or cell lysates were loaded onto a 96-well plate, and 100 μl reaction buffer (50 mM Tris–HCl, pH 7.5, 40 mM KCl, 5 mM $MgCl_2$, 0.5 mM ATP, and 0.5 mg/ml BSA) containing fluorogenic peptides were added to each aliquot. After incubation at 37°C for 15 min, fluorescence was measured with a plate reader equipped with 380-nm excitation and 450-nm emission filters. Proteasome

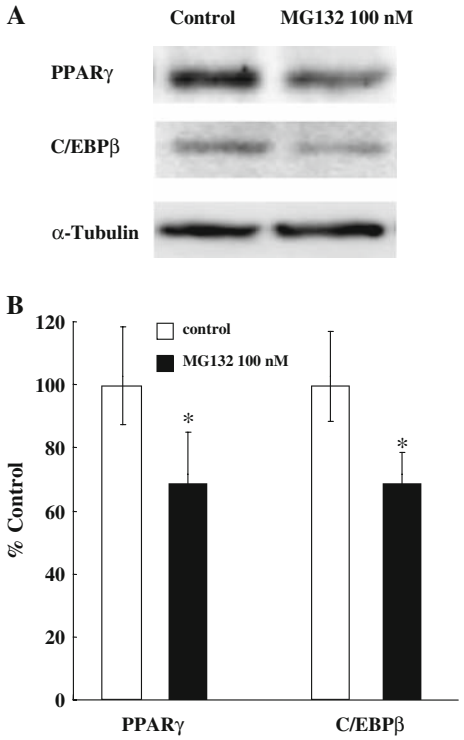

Fig. 4 Western immunoblot of PPARγ and C/EBPβ on 18th day after induction of differentiation. hADSC differentiation was induced into mature adipocytes as described in "Materials and methods" section with 100 nM MG132 for 18 days. **a** On the 18th day, cells were lysed, and 40 μg proteins were separated on 12% SDS-PAGE and subjected to immunoblotting with specific antibodies. The α-tubulin was used as a loading control. **b** Quantitative analysis was performed as indicated in "Materials and methods" section. Data are the means of three samples ± SD as a percentage of the control. * $P < 0.05$ significantly different from the control by unpaired *t* test

activity was calculated from the linear slope of a AMC (Sigma) standard curve.

Cell culture and cell viability assay

The hADSC was purchased from Lonza (Lonza Walkersville, Inc., Walkersville, MD, USA) and cultured in ADSC-BM medium (Lonza) with 10% fetal bovine serum (FBS), L-glutamine and antibiotics in 5% CO_2 at 37°C. To induce adipogenesis, hADSC cells (5×10^3 cells per cm^2) were plated in a culture dish and maintained for 5 days. Then, media were exchanged with Adipogenic Induction Medium (Lonza) for 3 days, followed by changing to Adipogenic Maintenance Medium (Lonza) for 3 days. Three cycles of induction/maintenance were then repeated. Cell viability was measured using 3-(4,5-dimethylthyazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Dojindo Laboratories, Kumamoto, Japan) assay. Proteasome inhibitor MG132 was obtained from Sigma.

Oil Red O staining

To determine lipid accumulation, cells were subjected to Oil Red O staining as described previously [30], with some modification. Briefly, after adipocyte differentiation, the cells were washed twice with phosphate-buffered saline (PBS), fixed with 10% buffered formalin, washed once with 60% isopropanol, and then stained with Oil Red O (5 mg/ml dissolved in 60% isopropanol) for 1 h at room temperature. After removing the staining solution, cells were washed twice with PBS, the dye retained in cells was eluted into 100% ethanol, and OD_{540} was determined.

Western blot analysis

hADSC cells were harvested, washed with PBS, and solubilized in 4 volumes of protein lysis buffer (50 mM Tris–HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, phosphatase inhibitor cocktail (Merck, Darmstadt, Germany), and 1 mM PMSF). Cells were disrupted by ultrasonication for 1 s for 20 times and centrifuged; the supernatant was used as a sample, and the resulting supernatants were kept at −80°C until protein analysis. Protein concentrations were determined using the Bradford assay. Proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using 12% polyacrylamide gel, then electrotransferred onto polyvinylidene difluoride membrane (Hybond-P) (GE Healthcare, Buckinghamshire, England). The membranes were blocked with TBS-T buffer (20 mM Tris–HCl, pH 7.6, 137 mM NaCl, and 0.1% Tween-20) with 5% Block Ace (DS Pharma Biomedical Co., Ltd., Osaka, Japan). To detect PPARγ and CCAAT/enhancer binding protein β (C/EBPβ), primary antibodies were mouse monoclonal anti-PPARγ (Perseus Proteomics Inc., Tokyo, Japan) and anti-C/EBPβ (Santa Cruz Biotechnology Inc., Santa Cruz, CA), while the secondary antibody was the anti-mouse IgG HRP (GE Healthcare), and proteins were visualized using the ECL detection system (GE Healthcare). Signal was detected by a luminescent image analyzer LAS-3000 mini (Fujifilm Corp., Tokyo, Japan). Protein levels were quantified from signal density values using the Science lab MultiGauge software (Fujifilm Corp.), normalized to the α-tubulin density values, and expressed as a percentage of the control.

Statistical analysis

Pearson's correlations were used to examine the correlations between proteasome activity and BMI, body fat, total cholesterol, and triglyceride. hADSC data were compared using either the unpaired *t* test or one-way ANOVA

followed by post hoc comparisons using Bonferroni's multiple comparison test. Statistical analyses were performed using SPSS version 13.0 (SPSS, Inc., Chicago, IL).

Acknowledgments This work was supported in part by Grants-in-Aid for Scientific Research from the Japan Science Technology Agency and the Core Research for Evolutional Science and Technology (CREST), 21st Century Center of Excellence (COE) Program.

References

1. E.A. Lew, L. Garfinkel, J. Chronic Dis. **32**, 563–576 (1979)
2. A. Tchernof, B. Lamarche, D. Prud'Homme, A. Nadeau, S. Moorjani, F. Labrie, P.J. Lupien, J.P. Després, Diabetes Care **19**, 629–637 (1989)
3. A.R. Dyer, P. Elliott, J. Hum. Hypertens. **3**, 299–308 (1989)
4. H.B. Hubert, M. Feinleib, P.M. McNamara, W.P. Castelli, Circulation **67**, 968–977 (1983)
5. B.A. Swinburn, I. Caterson, J.C. Seidell, W.P. James, Health Nutr. **7**, 123–146 (2004)
6. T.M. Frayling, N.J. Timpson, M.N. Weedon, E. Zeggini, R.M. Freathy, C.M. Lindgren, J.R. Perry, K.S. Elliott, H. Lango, N.W. Rayner, B. Shields, L.W. Harries, J.C. Barrett, S. Ellard, C.J. Groves, B. Knight, A.M. Patch, A.R. Ness, S. Ebrahim, D.A. Lawlor, S.M. Ring, Y. Ben-Shlomo, M.R. Jarvelin, U. Sovio, A.J. Bennett, D. Melzer, L. Ferrucci, R.J. Loos, I. Barroso, N.J. Wareham, F. Karpe, K.R. Owen, L.R. Cardon, M. Walker, G.A. Hitman, C.N. Palmer, A.S. Doney, A.D. Morris, G.D. Smith, A.T. Hattersley, M.I. McCarthy, Science **316**, 889–894 (2007)
7. C.T. Montague, I.S. Farooqi, J.P. Whitehead, M.A. Soos, H. Rau, N.J. Wareham, C.P. Sewter, J.E. Digby, S.N. Mohammed, J.A. Hurst, C.H. Cheetham, A.R. Earley, A.H. Barnett, J.B. Prins, S. O'Rahilly, Nature **387**, 903–908 (1997)
8. C. Fleury, M. Neverova, S. Collins, S. Raimbault, O. Champigny, C. Levi-Meyrueis, F. Bouillaud, M.F. Seldin, R.S. Surwit, D. Ricquier, C.H. Warden, Nat. Genet. **15**, 223–224 (1997)
9. T. Nakano, T. Shinka, M. Sei, Y. Sato, M. Umeno, K. Sakamoto, I. Nomura, Y. Nakahori, J. Med. Investig. **53**, 218–222 (2006)
10. N. Kubota, Y. Terauchi, H. Miki, H. Tamemoto, T. Yamaguchi, K. Komeda, S. Satoh, R. Nakaho, C. Ishii, T. Sugiyama, K. Eto, Y. Tsubamoto, A. Okuno, K. Murakami, H. Sekihara, G. Hasegawa, M. Naito, Y. Toyoshima, S. Tanaka, K. Shiota, T. Kitamura, T. Fujita, O. Ezaki, S. Aizawa, R. Nagai, K. Tobe, S. Kimura, T. Kadowaki, Mol. Cell **4**, 597–600 (1999)
11. K. Maeda, K. Okubo, I. Shimomura, T. Funahashi, Y. Matsuzawa, K. Matsubara, Biochem. Biophys. Res. Commun. **21**, 286–289 (1996)
12. T. Kawada, N. Takahashi, T. Fushiki, J. Nutr. Sci. Vitaminol. **47**, 1–12 (2001)
13. M.J. Kim, U.J. Chang, J.S. Lee, Mar. Biotechnol. **5**, 557–562 (2008)
14. A.M. Sharma, Int. J. Obes. **26**, S5–S7 (2002)
15. K. Sakamoto, Y. Sato, T. Shinka, M. Sei, I. Nomura, M. Umeno, A.A. Ewis, Y. Nakahori, Obesity **17**, 1044–1049 (2009)
16. K. Tanaka, C. Tsurumi, Mol. Biol. Rep. **24**, 3–11 (1997)
17. R.W. King, R.J. Deshaies, J.M. Peters, M.W. Kirschner, Science **274**, 1652–1659 (1996)
18. R.Z. Orlowski, Cell Death Differ. **6**, 303–313 (1999)
19. Wing, S.S. (2008). BMC Biochem. **9**, Suppl 1, S6
20. W.S. Kim, B.S. Park, J.H. Sung, J.M. Yang, S.B. Park, S.J. Kwak, J.S. Park, J. Dermatol. Sci. **48**, 15–24 (2007)
21. D. Noël, D. Caton, S. Roche, C. Bony, S. Lehmann, L. Casteilla, C. Jorgensen, B. Cousin, Exp. Cell Res. **314**, 1575–1584 (2008)
22. D.H. Lee, A.L. Goldberg, Trends Cell Biol. **8**, 397–403 (1998)
23. S. Tsubuki, H. Kawasaki, Y. Saito, N. Miyashita, M. Inomata, S. Kawashima, Biochem. Biophys. Res. Commun. **15**, 1195–1201 (1993)
24. T.L. Chang, C.L. Chang, W.Y. Lee, M.N. Lin, Y.W. Huang, K. Fan, Metabolism **58**, 1643–1648 (2009)
25. C. Wójcik, G.N. DeMartino, J. Biol. Chem. **277**, 6188–6197 (2002)
26. S. Meiners, D. Heyken, A. Weller, A. Ludwig, K. Stangl, P.M. Kloetzel, E. Krüger, J. Biol. Chem. **278**, 21517–21525 (2003)
27. A.F. Kisselev, A.L. Goldberg, Methods Enzymol. **398**, 364–378 (2005)
28. Q.Q. Tang, T.C. Otto, M.D. Lane, Proc. Natl. Acad. Sci. USA **100**, 850–855 (2003)
29. Q.Q. Tang, T.C. Otto, M.D. Lane, Proc. Natl. Acad. Sci. USA **100**, 44–49 (2003)
30. J.T. Tan, S.V. McLennan, W.W. Song, L.W. Lo, J.G. Bonner, P.F. Williams, S.M. Twigg, Am. J. Physiol. Cell Physiol. **295**, 740–751 (2008)