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Pioglitazone Treatment Enlarges Subcutaneous Adipocytes in Insulin-Resistant Patients

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Context: Obesity-related insulin resistance is associated with an increase in adipocyte size. In rodent models, treatment with the insulin-sensitizers thiazolidinediones (TZDs) leads to the appearance of small, insulin-sensitive adipocytes. Whether such TZD-dependent morphological changes occur in adipose tissue of insulin-resistant patients is unclear.

Objective: The objective of the study was to study the effects of treatment with the TZD pioglitazone on sc adipose tissue morphology and function in insulin-resistant subjects.

Design: This was a placebo-controlled, randomized crossover study.

Setting: The study was conducted at a university medical center.

Patients: Twelve adult patients with congenital adrenal hyperplasia (CAH) characterized by insulin resistance were included in this study.

Intervention: After a 4-wk run-in phase, patients were treated with pioglitazone (45 mg/d) followed by placebo, each for 16 wk or vice versa.

Main Outcome Measures: After both placebo and pioglitazone treatment, insulin sensitivity was determined by hyperinsulinemic euglycemic clamp and abdominal sc adipose tissue was obtained to measure adipocyte cell surface and expression of genes involved in glucose uptake and inflammation.

Results: Pioglitazone treatment significantly improved the insulin sensitivity index (placebo: $0.35 \pm 0.16 \mu\text{mol/kg} \cdot \text{min}$ per milliunit per liter; pioglitazone $0.53 \pm 0.16 \mu\text{mol/kg} \cdot \text{min}$ per milliunit per liter, $P < 0.001$) and increased mRNA expression levels of adiponectin and glucose transporter-4 in adipose tissue. The increase in insulin sensitivity was accompanied by a significant enlargement of the sc adipocyte cell surface (placebo: $2323 \pm 725 \mu\text{m}^2$; pioglitazone $2821 \pm 885 \mu\text{m}^2$, $P = 0.03$).

Conclusions: In the human situation, treatment of insulin-resistant subjects with pioglitazone improves insulin sensitivity, whereas at the same time, sc adipocyte cell surface increases. (*J Clin Endocrinol Metab* 94: 4453–4457, 2009)

Obesity is associated with an abnormal secretion pattern of adipocytes and increased infiltration of macrophages into adipose tissue (1, 2). Recent studies have shown that the increased risk for obese subjects to develop

insulin resistance and type 2 diabetes mellitus (T2DM) is associated with increased sc abdominal adipocyte surface (3, 4). Large adipocytes are relatively insulin insensitive and have an increased basal lipolysis and an altered

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in U.S.A.

Copyright © 2009 by The Endocrine Society
doi: 10.1210/jc.2009-0517 Received March 9, 2009. Accepted August 4, 2009.
First Published Online October 9, 2009

Abbreviations: BMI, Body mass index; CAH, congenital adrenal hyperplasia; GLUT-4, glucose transporter 4; ISI, insulin sensitivity index; MCP, monocyte chemoattractant protein; T2DM, type 2 diabetes mellitus; TZD, thiazolidinedione.

secretion of adipokines (5, 6). In addition, hypertrophic adipocytes promote the infiltration of macrophages into adipose tissue, leading to enhanced production of proinflammatory cytokines that contribute to the development of insulin insensitivity (7). It has therefore been assumed that the presence of large adipocytes may drive the development of insulin resistance.

Thiazolidinediones (TZDs) are an insulin-sensitizing class of pharmacological compounds widely used for treatment of T2DM, which exhibit their insulin-sensitizing effects by decreasing visceral fat content, enhancing insulin action, and improving glucose homeostasis (8). At the same time, TZDs increase the sc fat depot (9). TZDs are ligands for peroxisome proliferator-activated receptor- γ , a nuclear transcription factor highly expressed in adipose tissue (10). One of the adipose tissue-specific effects of TZDs, mainly described in rodent studies, is the stimulation of adipocyte differentiation and apoptosis of large adipocytes, resulting in an increased population of small, more insulin-sensitive adipocytes (11). This suggests that improvement in glucose homeostasis by TZD treatment will be accompanied by changes in adipocyte size.

In the present study, we investigated the effects of pioglitazone treatment on insulin sensitivity, sc adipocyte surface, and expression of genes involved in glucose uptake and inflammation, in insulin-resistant subjects.

Patients and Methods

Study population

Twelve patients with genetically characterized, classical, congenital adrenal hyperplasia (CAH) on a stable corticosteroid dose were studied in a randomized crossover design. Based on their underlying condition and chronic treatment, this group of patients is characterized by insulin resistance (12). After a 4-wk run-in phase, patients were randomized to treatments with placebo and pioglitazone (45 mg/d) in a blinded fashion for 16 wk or vice versa. At the end of each treatment period, sc adipose tissue biopsies were obtained under local anesthesia by needle biopsies 6–10 cm lateral to the umbilicus. Samples were taken after an overnight fast. Fasting plasma adiponectin, leptin, insulin, and glucose were determined and insulin sensitivity index (ISI) was assessed. Finally, the percentage of trunk fat was determined.

The ethical committee of the Radboud University Nijmegen Medical Centre approved the study protocol and the procedures followed were in accordance with institutional guidelines. All subjects gave written informed consent.

Adipocyte surface

Morphometry of individual fat cells was assessed in a blinded fashion using digital image analyses as described recently (13). In short, after biopsy, adipose tissue was immediately fixed in paraformaldehyde and embedded in paraffin. Subsequently paraffin

slides were stained with hematoxylin-eosin. Inasmuch our method to measure adipocyte size requires intact cell membranes, we initially optimized the thickness of our sections. Microscopic observation revealed that adipose tissue samples cut into sections less than 8 μm showed ruptured cells. To avoid multilayered cells, we used 10- μm sections for the analysis. For each subject, surface of all fat cells in four to seven microscopic fields of view were measured. On average, 250 fat cells were measured per specimen (range 150–350).

RNA isolation and real-time PCR analysis

Total RNA was extracted from sc adipose tissue using TRIzol reagent (Invitrogen, Carlsbad, CA). RNA concentration was determined using the NanoDrop (NanoDrop Technologies, Wilmington, MA). cDNA synthesis was performed using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). Real-time PCR was performed using Power-SYBR Green master mix and the 7300 real-time PCR system (Applied Biosystem, Warrington, UK). Expression of genes was normalized to $\beta 2\text{M}$ gene expression levels. Primer sequences are available on request.

Insulin sensitivity and body fat distribution

Insulin sensitivity was assessed by a hyperinsulinemic euglycemic clamp and expressed as glucose disposal rate and ISI essentially as described previously (14). Total-body dual-energy x-ray absorptiometry scanning was performed using a QDR 4500 densitometer (Hologic, Bedford, MA) to determine indirectly the percentage of trunk fat mass by dividing the absolute fat mass of the trunk by the total mass of the trunk.

Biochemical analyses

Glucose concentrations were measured using the oxidation method (Glucose Analyser 2; Beckman Instruments Inc., Fullerton, CA). Plasma concentrations of leptin and total adiponectin were determined using ELISAs (R&D Systems, Minneapolis, MN). The interassay and intraassay coefficients of variation were 6.5 and 3.5% for total adiponectin and 4.4 and 3.2% for leptin.

Statistical analyses

Variables are expressed as means \pm SD. Differences in adipocyte surface after placebo and pioglitazone treatment were studied using one-way univariate ANOVA. Student's paired *t* test was used to analyze statistical significance of treatment differences of other variables. Two-tailed $P < 0.05$ was considered significant. Tukey honestly significant difference *post hoc* testing was applied for multiple comparison testing. All statistics were performed using SPSS software (version 16.0; SPSS Inc., Chicago, IL).

Results

Study population characteristics

A total of 12 patients [five men, seven women aged 36 ± 9 yr; body mass index (BMI) 26.9 ± 4.7 kg/m²] completed the study. Mean fasting plasma insulin, glucose, triglyceride, and total cholesterol levels after placebo were 8.8 ± 5.8 mU/liter, 5.0 ± 0.2 mmol/liter, 0.96 ± 0.3 mmol/liter,

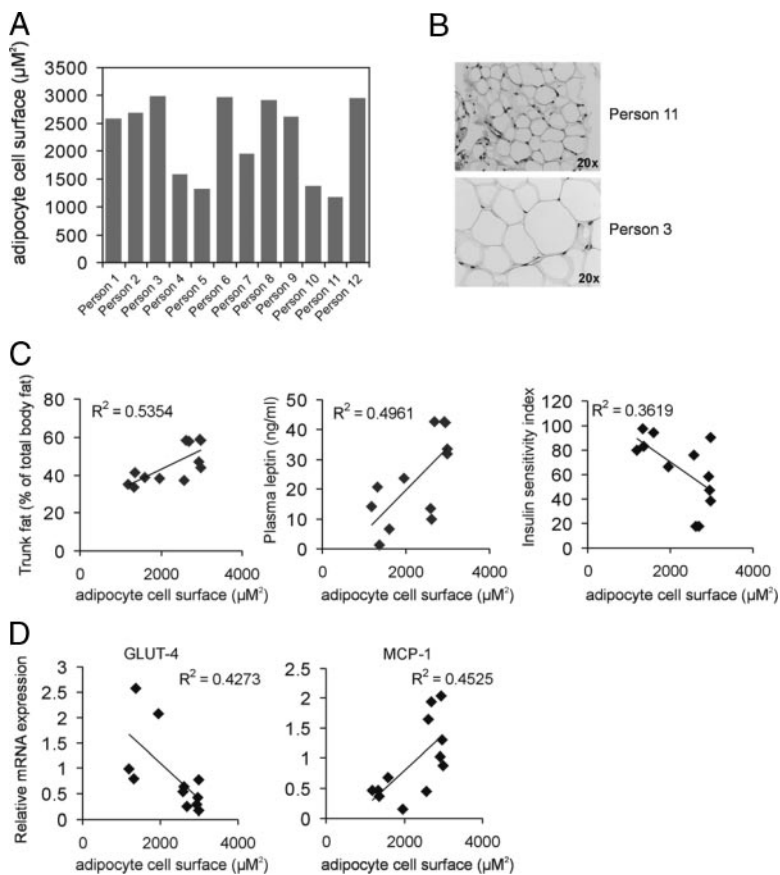


FIG. 1. Characteristics of sc adipocytes after placebo. A, Morphometry of fat cells assessed by digital image analysis. B, Hematoxylin-eosin staining images of sc adipose tissue of two subjects. C, Pearson correlation of mean adipocyte surface with percentage of trunk fat, plasma leptin level, and ISI. D, Gene expression levels of GLUT-4 and MCP-1.

and 4.53 ± 0.7 mmol/liter, respectively. The cell surface of sc adipocytes in our study population showed large variation. Whereas the mean sc adipocyte surface was $2323 \pm 725 \mu\text{m}^2$, clear interindividual differences were observed (Fig. 1, A and B). Interestingly, adipocyte cell surface was positively correlated with the percentage of trunk fat ($r = 0.73$; $P = 0.01$) and plasma leptin levels ($r = 0.70$; $P = 0.01$) and negatively correlated with the ISI ($r = -0.60$; $P = 0.04$) (Fig. 1C). In addition, adipocyte surface correlated negatively with glucose transporter 4 (GLUT-4) mRNA expression ($r = -0.65$; $P = 0.03$) and positively with the mRNA expression level of the inflammatory marker monocyte chemoattractant protein (MCP)-1 in the adipose tissue ($r = 0.57$; $P = 0.05$) (Fig. 1D). Noticeably, adjustment for BMI did not significantly alter the correlations that were observed between the different variables and adipocyte surface.

Effects of pioglitazone treatment

After 16 wk of pioglitazone treatment, body weight and BMI had increased (by 2.0 ± 3.3 kg and 0.7 ± 1.1 kg/m²; $P = 0.06$ for both), but no difference was observed in mean

trunk fat percentage. Insulin sensitivity improved significantly after pioglitazone as reflected by ISI (placebo: 0.35 ± 0.16 ; pioglitazone: $0.53 \pm 0.16 \mu\text{mol/kg} \cdot \text{min}$ per milliunit per liter), $P < 0.001$). The ISI was based on glucose disposal rate (placebo: $28.5 \pm 11.6 \mu\text{mol/kg} \cdot \text{min}$; pioglitazone $38.9 \pm 11.0 \mu\text{mol/kg} \cdot \text{min}$, $P < 0.001$) and the insulin level at 120 min (placebo: 87.5 ± 22.9 mU/liter; pioglitazone: 76.3 ± 13.8 mU/liter, $P = 0.03$). Subcutaneous adipocyte surface increased significantly (by $497 \pm 625 \mu\text{m}^2$; $P = 0.03$) after pioglitazone treatment: eight of 11 patients showed larger adipocytes (Fig. 2, A and B). Pioglitazone had no significant effect on fasting plasma insulin (7.9 ± 4.1 mU/liter after pioglitazone), glucose (5.1 ± 0.5 mmol/liter after pioglitazone), adiponectin (placebo: $5.4 \pm 3.9 \mu\text{g/ml}$; pioglitazone: $6.1 \pm 4.7 \mu\text{g/ml}$), and leptin levels (placebo: 23.6 ± 14.9 ng/ml; pioglitazone: 22.9 ± 16.3 ng/ml). Quantitative PCR analysis revealed increases in expression of adiponectin ($P < 0.01$) and GLUT-4 ($P = 0.10$) mRNA in adipose tissue after pioglitazone treatment. MCP-1 mRNA expression appeared lower after pioglitazone treatment, but the differences were not statistically significant (Fig. 2C). After pioglitazone treatment, the negative correlation between sc adipocyte surface and ISI was slightly weaker ($r = -0.58$; $P = 0.05$), and the negative correlation between GLUT-4 and sc adipocyte surface was no longer significant ($r = -0.42$; $P = 0.2$). Correlations between sc adipocyte surface and the percentage of trunk fat, plasma leptin levels, and MCP-1 gene expression were dramatically changed after pioglitazone treatment. In contrast to placebo treatment, the percentage trunk fat and plasma leptin levels were no longer positively correlated with sc adipocyte surface. In addition, the positive correlation of MCP-1 gene expression levels and adipocyte surface observed after placebo treatment was lost after pioglitazone treatment. Apparently, enlarged adipocyte surface no longer predicts metabolic abnormalities and adipose tissue functioning after pioglitazone treatment in this study population.

Discussion

In the present study, we describe the effect of pioglitazone on sc adipose tissue morphology, gene expression levels, and insulin sensitivity in a group of insulin-resistant patients. Although adipocyte hypertrophy was associated

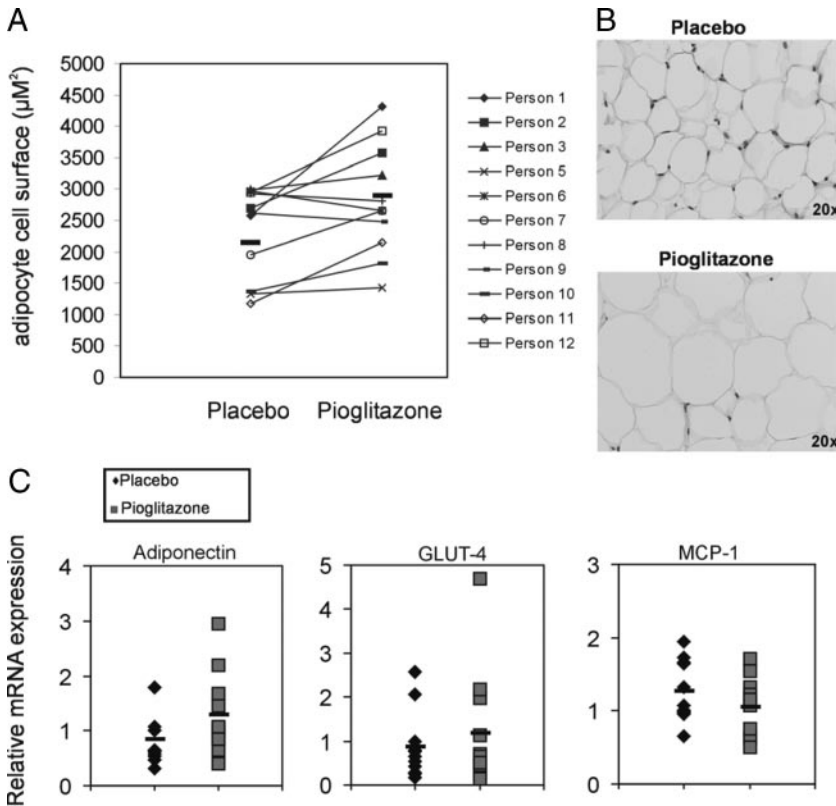


FIG. 2. Characteristics of sc adipocytes after 16 wk of pioglitazone treatment. A, Individual changes of mean sc adipocyte cell surface after pioglitazone treatment. B, Representative hematoxylin-eosin staining images of sc adipose tissue after placebo and pioglitazone treatment. C, Individual adipose tissue mRNA expression levels of adiponectin, GLUT-4, and MCP-1 after both treatments. Person 4 is missing because no biopsy was taken after pioglitazone treatment. Black dashes represent mean values.

with indices of insulin resistance, pioglitazone treatment resulted in an enlargement of sc adipocytes in parallel with an improvement of systemic insulin sensitivity.

The positive association between obesity-induced adipocyte hypertrophy and the development of insulin resistance and T2DM has been reported before (4). The development of hypertrophic adipocytes is partly explained by the impaired ability to differentiate new fat cells, resulting in enlargement of existing adipocytes (15). Enlarged adipocytes are characterized by a lower insulin-mediated glucose disposal, disturbances in lipid mobilization, and secretion of higher levels of leptin compared with smaller adipocytes (3, 16). In line with these detrimental effects of adipocyte hypertrophy, our data show that after placebo enlarged sc adipocyte surface correlates with elevated levels of circulating leptin, low insulin sensitivity, and lower GLUT-4 mRNA expression, an essential glucose uptake transporter. Adipocyte surface also positively correlates with secretion of proinflammatory adipokines (17). In our study, the MCP-1 proinflammatory gene expression positively correlated with sc adipocyte surface, suggesting that enlarged adipocytes have increased secretion levels of MCP-1 (17). Elevated MCP-1

levels might also be indicative of increased accumulation of macrophages in sc adipose tissue that promote the development of insulin resistance. Altogether our observations suggest that sc adipocyte hypertrophy is indicative of unfavorable metabolic functioning of adipose tissue after placebo treatment.

In agreement with previously published studies, treatment with pioglitazone significantly improved insulin sensitivity in our study population despite an increase in body weight (18). Because peroxisome proliferator-activated receptor- γ efficiently binds TZDs and is abundantly expressed in adipose tissue, pioglitazone is thought to primarily target the adipose tissue in which it promotes the development of small adipocytes characterized by an improvement in metabolic function. However, evidence in support of this mechanism of action has mainly been obtained from rodent studies (11). The number of studies that have investigated sc adipocyte surface in response to TZD treatment in humans is limited and has revealed ambiguous results. So far, studies have reported no change or an increase in the number of small adipocytes together with an improvement of insulin sensitivity after TZD treatment (19, 20), although a trend toward fat cell volume increase was also observed (21). In the present study, we observed a clear increase in sc adipocyte surface by pioglitazone together with an improvement of whole-body insulin sensitivity. These divergent results may be explained by differences in methodology. Previous studies measured adipocyte surface using isolated adipocytes obtained after collagenase digestion and subsequent centrifugation. This treatment usually results in breakage of large cells, leading to an unrepresentative sample of the original cell population with underestimation of large cells (22). In the present study, immediate fixation of adipose tissue biopsies after isolation warranted accurate estimation of adipocyte surfaces.

Importantly, positive correlations between adipocyte surface and plasma leptin, percentage trunk fat, and MCP-1 gene expression levels, markers indicative of insulin resistance and adipose tissue dysfunctioning, disappeared after pioglitazone-induced enlargement of adipocytes. These findings suggest that pioglitazone-induced adipocyte surface enlargement does not have a detrimental

but a beneficial effect on adipose tissue metabolism. Hypothetically the pioglitazone-induced adipocyte hypertrophy might result from redistribution of visceral and ectopic fat toward sc storage, ultimately leading to smaller visceral, although enlarged, sc adipocytes. Because no visceral fat biopsies were taken, no data are available to support this hypothesis.

This study was performed in a group of adult patients with CAH on stable corticosteroid treatment. These patients are characterized by insulin resistance (12) and were used in this study as a human model for obesity-associated insulin resistance. The advantage of this approach is that pioglitazone treatment will not lead to changes in glucose levels, and hence, the pure, not confounded by changes in glycemic level, effect of the TZD on fat metabolism can be studied. The improvement of insulin sensitivity suggests a normal response to pioglitazone in CAH patients and affirms the applicability of the model. However, we cannot exclude that other human models of insulin resistance would show different responses.

In conclusion, our data, obtained from human adipose tissue samples, confirm that increased sc adipocyte surface correlates with indices of insulin resistance at the whole-body and cellular level. Pioglitazone treatment enlarges adipose tissue and increases adipocyte surface, whereas at the same time improving the systemic insulin sensitivity.

Acknowledgments

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Disclosure Summary: The authors have nothing to disclose.

References

- Iacobellis G, Ribaldo MC, Zappaterreno A, Iannucci CV, Leonetti F 2005 Prevalence of uncomplicated obesity in an Italian obese population. *Obes Res* 13:1116–1122
- Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante Jr AW 2003 Obesity is associated with macrophage accumulation in adipose tissue. *J Clin Invest* 112:1796–1808
- Larson-Meyer DE, Heilbronn LK, Redman LM, Newcomer BR, Frisard MI, Anton S, Smith SR, Alfonso A, Ravussin E 2006 Effect of calorie restriction with or without exercise on insulin sensitivity, β -cell function, fat cell size, and ectopic lipid in overweight subjects. *Diabetes Care* 29:1337–1344
- Weyer C, Foley JE, Bogardus C, Tataranni PA, Pratley RE 2000 Enlarged subcutaneous abdominal adipocyte size, but not obesity itself, predicts type II diabetes independent of insulin resistance. *Diabetologia* 43:1498–1506
- Kahn BB, Flier JS 2000 Obesity and insulin resistance. *J Clin Invest* 106:473–481
- Reardon MF, Goldrick RB, Fidge NH 1973 Dependence of rates of lipolysis, esterification, and free fatty acid release in isolated fat cells on age, cell size, and nutritional state. *J Lipid Res* 14:319–326
- Di Gregorio GB, Yao-Borengasser A, Rasouli N, Varma V, Lu T, Miles LM, Ranganathan G, Peterson CA, McGehee RE, Kern PA 2005 Expression of CD68 and macrophage chemoattractant protein-1 genes in human adipose and muscle tissues: association with cytokine expression, insulin resistance, and reduction by pioglitazone. *Diabetes* 54:2305–2313
- Yang X, Smith U 2007 Adipose tissue distribution and risk of metabolic disease: does thiazolidinedione-induced adipose tissue redistribution provide a clue to the answer? *Diabetologia* 50:1127–1139
- Mori Y, Murakawa Y, Okada K, Horikoshi H, Yokoyama J, Tajima N, Ikeda Y 1999 Effect of troglitazone on body fat distribution in type 2 diabetic patients. *Diabetes Care* 22:908–912
- Vidal-Puig AJ, Considine RV, Jimenez-Liñan M, Werman A, Pories WJ, Caro JF, Flier JS 1997 Peroxisome proliferator-activated receptor gene expression in human tissues. Effects of obesity, weight loss, and regulation by insulin and glucocorticoids. *J Clin Invest* 99:2416–2422
- Okuno A, Tamemoto H, Tobe K, Ueki K, Mori Y, Iwamoto K, Umesono K, Akanuma Y, Fujiwara T, Horikoshi H, Yazaki Y, Kadowaki T 1998 Troglitazone increases the number of small adipocytes without the change of white adipose tissue mass in obese Zucker rats. *J Clin Invest* 101:1354–1361
- Charmandari E, Weise M, Bornstein SR, Eisenhofer G, Keil MF, Chrousos GP, Merke DP 2002 Children with classic congenital adrenal hyperplasia have elevated serum leptin concentrations and insulin resistance: potential clinical implications. *J Clin Endocrinol Metab* 87:2114–2120
- Stienstra R, Duval C, Keshtkar S, van der LJ, Kersten S, Müller M 2008 Peroxisome proliferator-activated receptor γ activation promotes infiltration of alternatively activated macrophages into adipose tissue. *J Biol Chem* 283:22620–22627
- Bredie SJ, Tack CJ, Smits P, Stalenhoef AF 1997 Nonobese patients with familial combined hyperlipidemia are insulin resistant compared with their nonaffected relatives. *Arterioscler Thromb Vasc Biol* 17:1465–1471
- Danforth Jr E 2000 Failure of adipocyte differentiation causes type II diabetes mellitus? *Nat Genet* 26:13
- Couillard C, Mauriège P, Imbeault P, Prud'homme D, Nadeau A, Tremblay A, Bouchard C, Després JP 2000 Hyperleptinemia is more closely associated with adipose cell hypertrophy than with adipose tissue hyperplasia. *Int J Obes Relat Metab Disord* 24:782–788
- Skurk T, Alberti-Huber C, Herder C, Hauner H 2007 Relationship between adipocyte size and adipokine expression and secretion. *J Clin Endocrinol Metab* 92:1023–1033
- Miyazaki Y, Mahankali A, Wajsborg E, Bajaj M, Mandarino LJ, DeFronzo RA 2004 Effect of pioglitazone on circulating adipocytokine levels and insulin sensitivity in type 2 diabetic patients. *J Clin Endocrinol Metab* 89:4312–4319
- Hammarstedt A, Sotasakis VR, Gogg S, Jansson PA, Smith U 2005 Improved insulin sensitivity and adipose tissue dysregulation after short-term treatment with pioglitazone in non-diabetic, insulin-resistant subjects. *Diabetologia* 48:96–104
- Shadid S, Jensen MD 2003 Effects of pioglitazone versus diet and exercise on metabolic health and fat distribution in upper body obesity. *Diabetes Care* 26:3148–3152
- Ciaraldi TP, Kong AP, Chu NV, Kim DD, Baxi S, Loviscach M, Plodkowski R, Reitz R, Caulfield M, Mudaliar S, Henry RR 2002 Regulation of glucose transport and insulin signaling by troglitazone or metformin in adipose tissue of type 2 diabetic subjects. *Diabetes* 51:30–36
- Hirsch J, Gallian E 1968 Methods for the determination of adipose cell size in man and animals. *J Lipid Res* 9:110–119