Regional Differences in the Response of Human Pre-Adipocytes to PPARγ and RXRα Agonists

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We have previously reported that omental (OM) preadipocytes respond less well to the prodifferentiating effects of thiazolidinediones than do preadipocytes from subcutaneous (SC) depots. This finding is consistent with in vivo alterations in fat distribution that occur in humans treated with thiazolidinediones. To explore these site-related differences further, we used real-time RT-PCR to quantify the specific mRNAs encoding peroxisome proliferator-activated receptor (PPAR) γ1 and γ2 and found that both isoforms were more highly expressed in SC than in OM preadipocytes. After 10 days of thiazolidinedione treatment, preadipocytes from both depots showed a small and comparable increase in expression of PPARγ1 mRNA (1.7 ± 0.2-fold [*P* = 0.007]) and 1.3 ± 0.1-fold [*P* = 0.008] increase for SC and OM, respectively). There was a much larger increase in PPARγ2 expression, which was significantly greater in SC compared with OM preadipocytes (11.1 ± 2.8-fold [*P* = 0.0003] and 5.5 ± 1.7-fold [*P* = 0.0003], respectively; *P* = 0.014 for SC versus OM). To establish whether the refractoriness of OM preadipocytes to differentiation was unique to activators of the PPARγ pathway, we examined the effects of the retinoid X receptor (RXR) ligand LG100268. As assessed by glycerol-3-phosphate dehydrogenase activity, LG100268 had a greater effect on the differentiation of SC compared with OM preadipocytes when examined alone (SC = 5.7 ± 1.7-fold vs. OM = 1.9 ± 0.6-fold; *P* < 0.05) or in combination with rosiglitazone (SC = 27.0 ± 7.5 vs. OM = 10.6 ± 3.6-fold; *P* < 0.05). Consistent with this, RXRα mRNA levels were also higher in SC than in OM preadipocytes. In summary, the previously reported insensitivity of OM preadipocytes to the differentiating effects of thiazolidinediones may relate to their lower basal levels of PPARγ1 and γ2 mRNA and their diminished capacity to upregulate PPARγ2 expression in response to ligand. That omentally derived cells also show reduced responsiveness to the prodifferentiating actions of an RXR ligand and a lower expression of RXRα in the undifferentiated state suggests that they may have a more generalized resistance to differentiation. *Diabetes* 51:718–723, 2002

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DM, differentiating medium; OM, omental; PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator response element; RXR, retinoid X receptor; SC, subcutaneous.

In the 1950s, Vague et al. (1) provided the first formal evidence that the anatomical distribution of human body fat may have important metabolic implications. They noted that upper body (android) obesity, which is associated with increased omental (OM) adiposity, was frequently found in individuals who had diabetes and atherosclerosis. In contrast, lower body (gynoid) obesity, associated with increased subcutaneous (SC) adiposity, was less closely linked to these health problems. Subsequently, a substantial body of clinical, epidemiological, and physiological evidence has accumulated to support the notion that OM adiposity is particularly deleterious to health (2–8). These observations have prompted research into the intrinsic biological differences in adipose tissue from both the OM and SC depots. OM adipocytes have been shown to have higher rates of triglyceride turnover compared with those derived from the SC depot (9). This has been attributed to an increased sensitivity to the lipolytic actions of catecholamines (10–12) and a decreased sensitivity to the antilipolytic effects of insulin (13,14).

Recently, the thiazolidinedione class of compounds has been developed for the treatment of type 2 diabetes (15). These compounds are ligands for the peroxisome proliferator-activated receptor γ (PPARγ) (16,17). Although the precise mechanism of action of the thiazolidinedione compounds is unknown, it has been shown that the activation of PPARγ is closely linked to their antihyperglycemic effects (18,19). Patients who are treated with thiazolidinediones show a change in body fat distribution with an increase in the SC/OM ratio (20). Given the deleterious effects of OM adiposity, this is thought to be one of the mechanisms by which these compounds increase insulin sensitivity. We have previously shown that in vitro, the thiazolidinedione (rosiglitazone) promotes adipogenesis of SC preadipocytes to a greater extent than preadipocytes derived from the OM depot (21). However, the precise mechanism for the depot-specific effects of the thiazolidinediones has not been identified. To address this question specifically, we measured, using quantitative real-time PCR, the expression of both PPARγ1 and γ2 mRNA levels in preadipocytes from both depots both in the undifferentiated state and in response to rosiglitazone. Synthetic agonists for retinoid X receptorα (RXRα) that also show insulin-sensitizing properties in vivo have been developed (22). Given the role of RXRα in adipogenesis, as the obligate binding partner for PPARγ, we also examined the expression of RXRα in preadipocytes from the two depots and the effect of the synthetic RXRα agonist

LG100268 on OM and SC preadipocyte differentiation in vitro.

RESEARCH DESIGN AND METHODS

Participants and sample acquisition. OM and SC adipose tissue biopsies were obtained from patients who were undergoing elective open-abdominal surgery. All patients fasted for at least 6 h preoperatively, and all underwent general anesthesia. None of the patients had diabetes or severe systemic illness. The patient group included 17 participants, 12 women (age 52 ± 14 years, BMI 27.3 ± 5.9 kg/m^2) and 5 men (age 61 ± 3 years, BMI 28.1 ± 5.6 kg/m^2). Cambridge Local Research Ethics Committee approval was obtained, and all patients involved gave informed consent.

Preadipocyte isolation and cell culture. Adipose tissue biopsies were placed in normal saline and immediately processed (transport time to the laboratory was 5 min). The adipose tissue was diced finely and digested in a collagenase solution (Hanks balanced salt solution containing 3 mg/ml type II collagenase (Sigma, St. Louis, MO) and 1.5% BSA) for 1 h in a shaking water bath at 37°C. After digestion, the mature adipocytes were separated from the stromovascular cells by centrifugation (10 min, 1,500*g*) of the digestion mixture over dionyl-phthalate oil. The stromovascular pellet containing the preadipocytes was treated with erythrocyte lysis solution (154 mmol/l NH_4Cl, 10 mmol/l $KHCO_3$, 0.1 mmol/l EDTA) for 5 min at room temperature and centrifuged (5 min, 1,500*g*). The preadipocyte pellet was cultured in DME/Ham's F12 medium supplemented with 10% FBS, 2 mmol/l glutamine, 100 units of penicillin, and 0.1 mg/ml streptomycin at 37°C in a humidified 95% air and 5% CO_2 incubator. Cultures were passaged four times and grown to confluence (day 0). At confluence, the medium was changed to a serum-free hormonally modified differentiating medium (DM) consisting of DME/Ham's F12 supplemented with 2 mmol/l glutamine, 100 units of penicillin, 0.1 mg/ml streptomycin, 33 μmol/l Biotin, 17 μmol/l pantothenic acid, 10 μg/ml human apotransferrin, 0.2 nmol/l tri-iodothyronine, 100 nmol/l cortisol, and 500 nmol/l insulin. The DM was supplemented with rosiglitazone (10^{-7} mol/l), LG100268 (10^{-7} mol/l), rosiglitazone (10^{-7} mol/l) and LG100268 (10^{-7} mol/l), or vehicle (DMSO). For the first 3 days only, 250 μmol/l iso-butylmethylxanthine was added to the DM. At various time points postdifferentiation, total RNA, and protein was extracted. The extent of differentiation was measured morphologically (intracellular lipid accumulation) and biochemically by measuring glycerol-3-phosphate dehydrogenase (G3PDH) activity as outlined.

RNA extraction. Total RNA was extracted using the RNeasy mini extraction kit (Qiagen, West Sussex, U.K.) according to the manufacturer's recommendations. RNA samples were quantified by spectrophotometry, and integrity was assessed by agarose gel electrophoresis and ethidium bromide staining. The RNA samples were then diluted as appropriate in RNase-free water and stored at −80°C until use.

G3PDH activity assay. Preadipocytes cultured in 24-well plates were washed in PBS (pH 7.4), harvested by scraping in 200 μl of ice-cold 50 mmol/l Tris-HCl (pH 7.5) containing 1 mmol/l EDTA and 500 μmol/l DL-DTT, and transferred to prechilled microtubes. Cells were disrupted by sonication and then centrifuged at 12,000*g* for 15 min at 4°C. The supernatant was then assayed for G3PDH activity in a final concentration of 100 mmol/l triethanolamine-HCl (pH 7.5), 2.5 mmol/l EDTA, 0.12 mmol/l NADH, 50 μmol/l DL-DTT, and 0.2 mmol/l dihydroxyacetone phosphate. The absorbance at 340 nm was monitored in a Monarch analyzer (Instrumentation Laboratory, Milan, Italy) at 37°C at 30-s intervals for 3 min. An aliquot of the supernatant was assayed for protein concentration using the Coomassie Plus protein assay reagent (Pierce, Tattenhall, U.K.). Units of measurement were milliunits per milligram of supernatant protein, 1 mU of enzyme activity being the amount catalyzing the oxidation of 1 nmol NADH/min.

Quantification of mRNA expression by real-time quantitative PCR. Total RNA (5 μg) was reverse-transcribed for 1 h at 37°C in a 25-μl reaction containing 1× RT buffer (50 mmol/l Tris-HCl, 75 mmol/l KCl, 3 mmol/l $MgCl_2$, and 10 mmol/l DTT), 2.5 μg of random hexamers, 2 mmol/l dNTPs, and 200 units of M-MLV (Moloney murine leukemia virus) reverse transcriptase (Promega, Southampton, U.K.). Reactions in which RNA was omitted served as negative controls.

A reaction containing 5 μg adipocyte total RNA was also included as a standard. After first-strand cDNA synthesis, this standard was serially diluted one in two in DNase-free water to generate a standard curve for the PCR analysis.

Oligonucleotide primers and taqman probes were designed using Primer Express, version 1.0 (Perkin-Elmer Applied Biosystems, Foster City, CA) and sequences from the GeneBank database (accession numbers X90563, U63415, and NM002957). For quantification of PPARγ1 and -γ2 isoforms, the same reverse primer and fluorogenic probe were used but different forward primers. The sequences were as follows: PPARγ1 forward 5′GTGGCCGCAGAAATGACC3′, PPARγ2 forward: 5′GATACACTGTCTGCAAACATATCACAA3′; reverse: 5′CCACGGAGCTGATCCCAA3′; and probe: 5′AGAGATGCCATTCTGGCCCACCAACTT3′. For quantification of RXRα mRNA, the primer/probe sequences were as follows: RXRα forward: 5′AGGCCTACTGCAAGCACAAGTAC3′; RXRα reverse: 5′GGCAGGCGGAGCAAGAG3′; and RXRα probe: 5′CAGAGCAGCCGGGAAGGTTCGC3′. The taqman probes were labeled at the 5′ end with the reporter dye FAM (6-carboxy-fluorescein) and at the 3′ end with the quencher TAMRA (6-carboxy-tetramethyl-rhodamine). Oligonucleotide primers and taqman probes for the GAPDH internal control were purchased from Perkin-Elmer.

PCR was carried out in duplicate for each sample on an ABI 7700 sequence detection system (Perkin-Elmer Biosystems). Each 25-μl reaction contained 2 μl of first-strand cDNA, 1× PCR master mix, 300 nmol/l of each forward and reverse primer, and 75 nmol/l taqman probe. All reactions were carried out using the following cycling parameters: 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. After PCR, standard curves were constructed from the standard reactions for each target gene and internal control by plotting Ct values, i.e., the cycle number at which the fluorescence signal exceeds background, versus log cDNA dilution. The Ct readings for each of the unknown samples were then used to calculate the amount of either target or internal control relative to the standard. For each sample, results were normalized by dividing the amount of target by the amount of internal control. Because the amplification efficiency of PPARγ1 and γ2 was equal, the γ2/γ1 ratio could be calculated using the equation $2^{-\Delta\Delta Ct}$, where ΔΔCt = γ2 − γ1 Ct. Intra- and interassay coefficients of variation were 0.03–2.21% and 0.03–2.42%, respectively.

Statistical analysis. Intraindividual comparisons were analyzed using the paired Wilcoxon nonparametric test. Interindividual comparisons were analyzed using the unpaired Wilcoxon test. Linear correlations were analyzed using the Pearson test. P values <0.05 were considered significant.

RESULTS

PPARγ and RXRα mRNA expression in OM and SC preadipocytes. PPARγ1, -γ2, and RXRα mRNAs were measured in confluent, undifferentiated SC and OM preadipocytes using real-time quantitative PCR. Both PPAR isoforms were more highly expressed in SC preadipocytes compared with those derived from the OM depot (SC = 3.30 ± 0.49 versus. OM = 2.18 ± 0.26 and SC = 1.15 ± 0.29 versus OM = 0.55 ± 0.11 arbitrary units for PPARγ1 [Fig. 1*A*] and -γ2 [Fig. 1*B*], respectively; both $P < 0.01$). PPARγ1 was by far the most abundant isoform in both depots. However the γ2/γ1 ratio was significantly higher in SC preadipocytes compared with OM preadipocytes (0.02 ± 0.005 vs. 0.01 ± 0.002 respectively; $P < 0.01$; Fig. 1*C*). In contrast, similar levels of both γ1 and γ2 were found in isolated mature adipocytes from both depots (data not shown).

RXRα mRNA expression was also significantly higher in SC preadipocytes compared with those derived from the OM depot (0.12 ± 0.03 vs. 0.067 ± 0.01 arbitrary units; P = 0.002; Fig. 2).

Effects of rosiglitazone on PPARγ mRNA expression. PPARγ1 and γ2 mRNA levels were quantified in SC and OM preadipocytes that were induced to undergo differentiation for 10 days in the presence or absence of rosiglitazone (Fig. 3). Rosiglitazone treatment resulted in a small increase in PPARγ1 mRNA (1.66 ± 0.21-fold [P = 0.007] and 1.34 ± 0.14-fold [P = 0.008] relative to DM alone for SC and OM preadipocytes, respectively). Rosiglitazone produced a much greater increase in PPARγ2 mRNA levels in preadipocytes from both depots. This increase in PPARγ2 mRNA was significantly greater in SC preadipocytes compared with OM preadipocytes (11.11 ± 2.80 and 5.53 ± 1.74-fold relative to DM alone, respectively; P = 0.0003 for both and P = 0.014 for the difference between OM and SC). To determine the relationship between PPARγ isoform expression and human adipocyte differen-

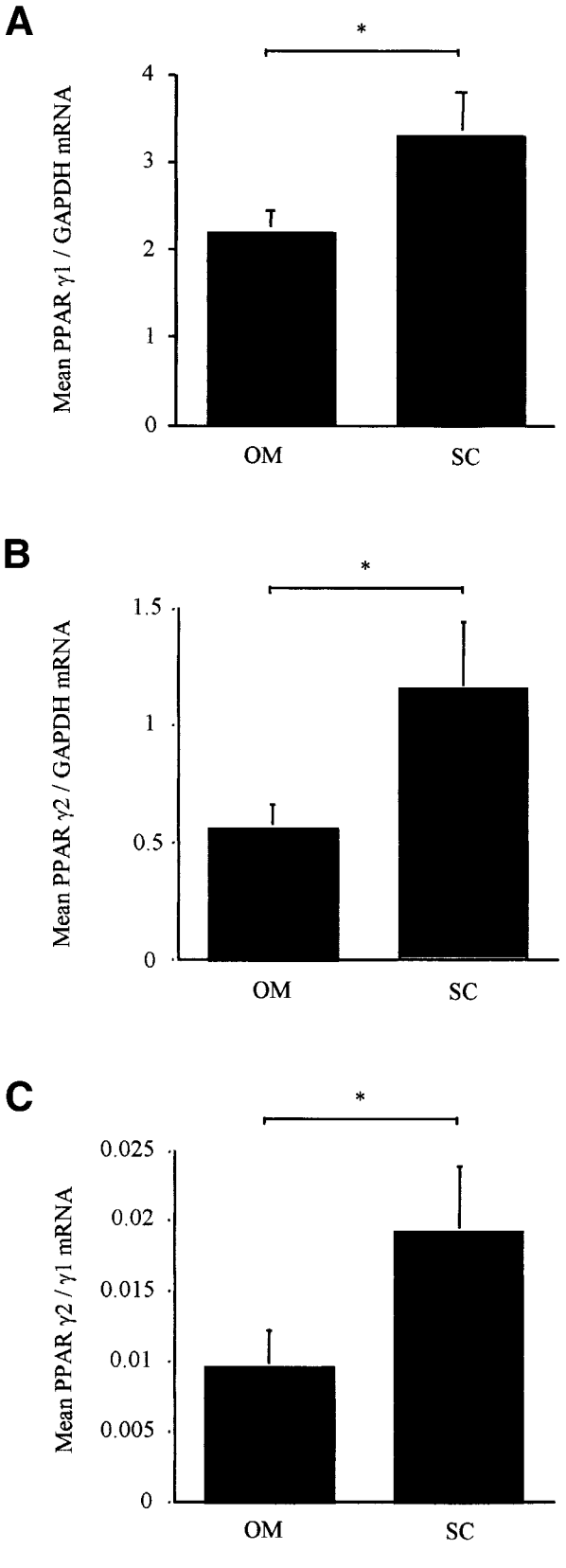

FIG. 1. PPARγ1′2fGAPDH (*A*), PPARγ2/GAPDH (*B*), and γ2/γ1 (*C*) mRNA expression in human OM and SC preadipocytes grown to confluence in vitro (undifferentiated). Results shown are mean ± SE from 15 participants; *P = 0.008.

tiation (measured as G3PDH enzyme activity), we undertook a detailed time-course study in SC preadipocytes

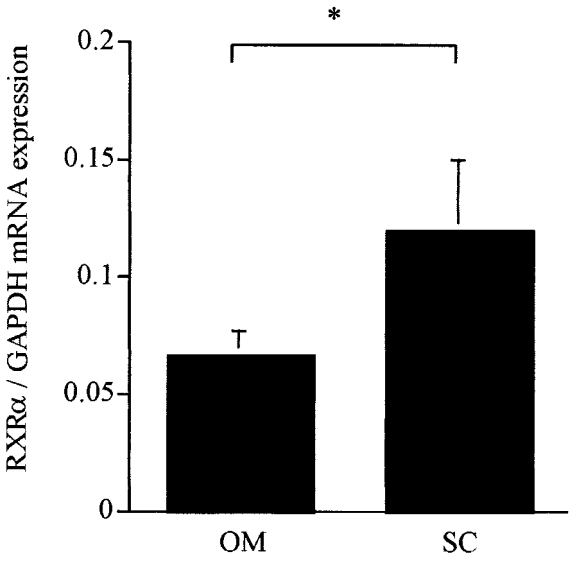

FIG. 2. RXRα/GAPDH mRNA expression in human OM and SC preadipocytes grown to confluence in vitro (undifferentiated). Results shown are mean ± SE from 12 participants; *P = 0.002.

from a single participant. There was an extremely strong correlation between both PPARγ1 and -γ2 expression and G3PDH enzyme activity over the course of this experiment (G3PDH versus γ1, r = 1.0, P = 0.002; G3PDH versus γ2, r = 1.0, P = 0.003; Fig. 4). However, as a result of the poor levels of differentiation, no significant correlation between differentiation and PPARγ mRNA expression was found in OM preadipocytes.

Effects of an RXR agonist on the differentiation of preadipocytes. To establish whether the refractoriness of OM preadipocytes to differentiation was unique to activators of the PPARγ pathway, we examined the effects of the RXR ligand LG100268. LG100268 treatment promoted preadipocyte differentiation as assessed by microscopic fat accumulation (Fig. 5) and measurements of G3PDH enzyme activity (Fig. 6). The enhancement of G3PDH activity was significantly lower than that seen with rosiglitazone. As with the PPARγ agonist, the RXR agonist also showed depot specificity (5.7 ± 1.7 and 1.9 ± 0.6-fold increases in G3PDH relative to DM alone for SC and OM, respectively; $P < 0.05$). PPARγ and RXR ligands were synergistic with the combination producing a 27 ± 7-fold

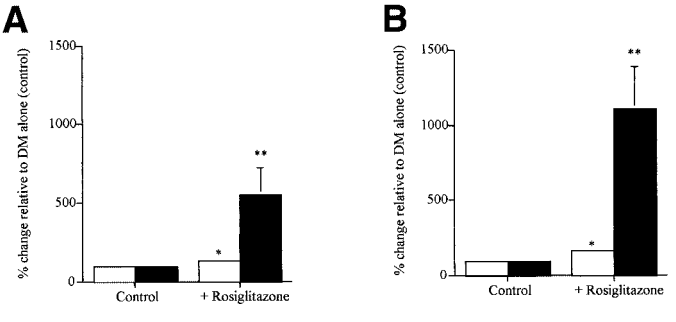

FIG. 3. PPARγ1 (□) and γ2 (■) mRNA expression in human OM (*A*) and SC (*B*) preadipocytes after 10 days postdifferentiation in DM + vehicle (control) or DM + rosiglitazone (10^{-7} mol/l). Results shown are mean percentage of control ± SE from 17 participants; *P < 0.01, **P < 0.001. P = 0.014 for difference in fold increase of PPARγ2 between OM and SC.

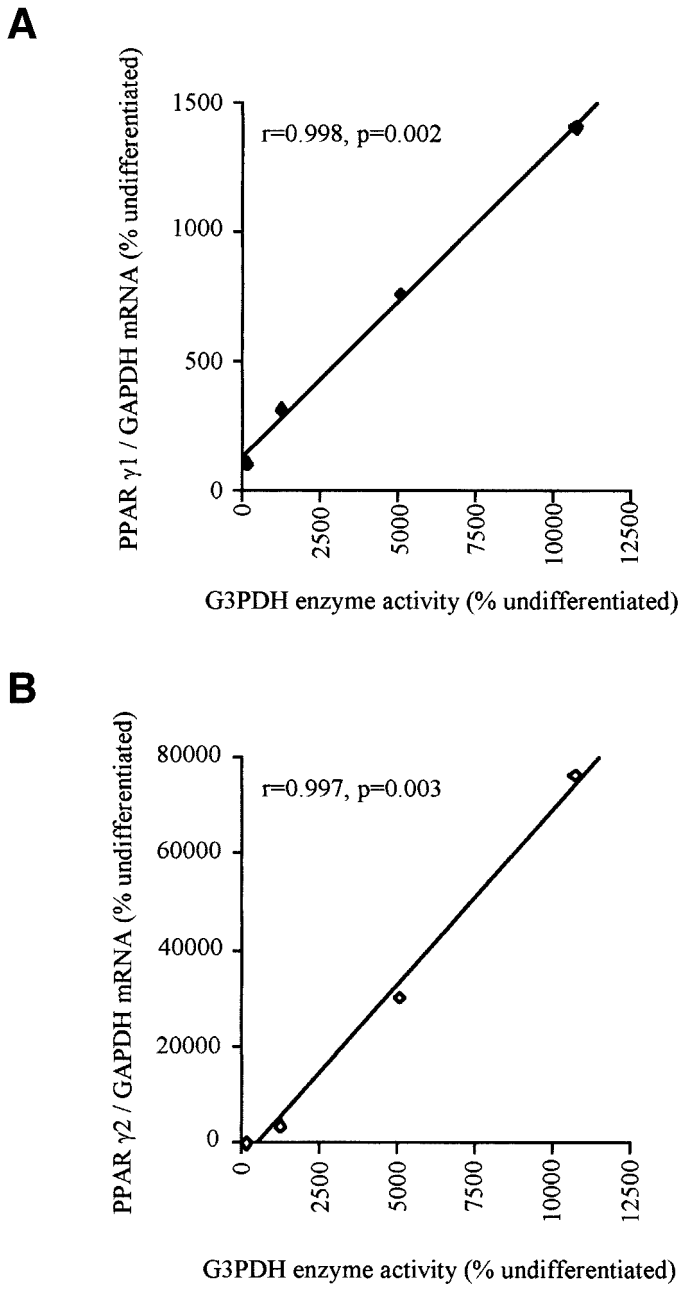

FIG. 4. Relationship between human SC preadipocyte G3PDH enzyme activity and either PPARγ1 (*A*) or PPARγ2 (*B*) mRNA expression at 5, 10, and 15 days postdifferentiation. Results are expressed as percentage of day 0 (undifferentiated).

increase in G3PDH levels in SC preadipocytes and an 11 ± 4-fold increase in OM preadipocytes compared with DM alone.

DISCUSSION

The thiazolidinedione class of compounds is high-affinity ligands for the nuclear receptor PPARγ, which have clinically useful actions as insulin sensitizers and antihyperglycemic agents in humans (15,16). Because obesity is commonly associated with insulin resistance, it seems somewhat paradoxical that agents that can promote adipogenesis have beneficial metabolic effects. In this regard, we have previously shown that thiazolidinediones promote the differentiation of human preadipocytes derived

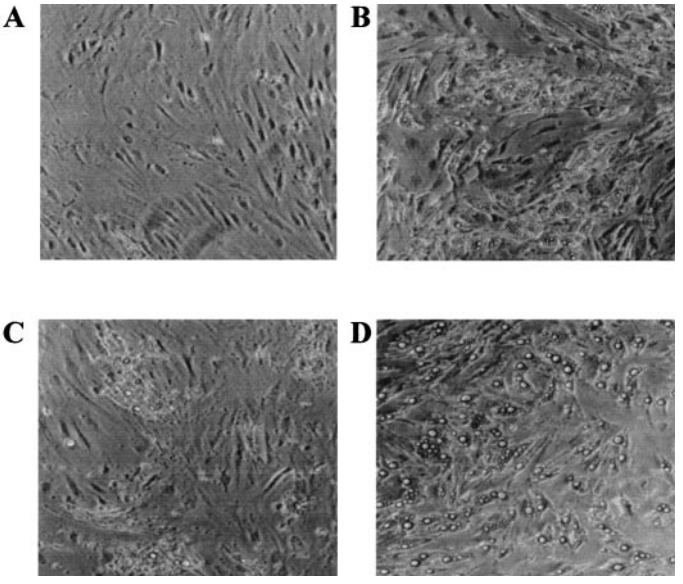

FIG. 5. Cellular morphology of human SC preadipocytes cultured for 15 days in DM + vehicle (*A*), DM + rosiglitazone (10^{-7} mol/l; *B*), DM + LG100268 (10^{-7} mol/l; *C*), or DM + rosiglitazone (10^{-7} mol/l) and LG100268 (10^{-7} mol/l; *D*).

from SC sites to a greater extent than those derived from the OM depot (21). This finding is consistent with in vivo studies that have reported that patients who receive thiazolidinedione show a redistribution of body fat from the OM to the SC depot (20). Given the specific deleterious effects of OM adiposity on insulin sensitivity, this change in body fat distribution could represent one of the mechanisms by which the thiazolidinediones mediate their insulin-sensitizing effects in vivo.

These data raise the question of why OM preadipocytes seem less responsive to the prodifferentiating effects of

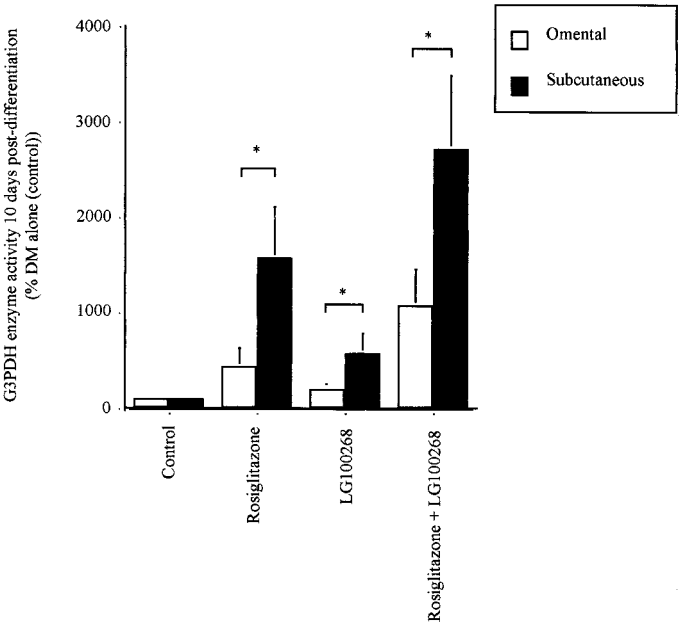

FIG. 6. G3PDH enzyme activity in human OM and SC preadipocytes differentiated for 10 days in DM + vehicle (control), DM + rosiglitazone (10^{-7} mol/l), DM + LG100268 (10^{-7} mol/l), or DM + rosiglitazone (10^{-7} mol/l and LG100268 (10^{-7}mol/l). Results shown are mean percentage of control ± SE from 14 participants, *P = 0.04.

thiazolidinediones than subcutaneously derived cells. In our original studies, gross levels of PPARγ protein were similar in OM and SC preadipocytes (21). In this study, we examined PPARγ mRNA levels using highly sensitive real-time quantitative RT-PCR assays that distinguish between the two major isoforms, γ1 and γ2. The importance of examining the two isoforms is exemplified by the fact that PPARγ2, although of lower abundance, is a highly fat cell–specific isoform and is much more tightly regulated by nutritional manipulation (23). Using this methodology, we found both PPARγ1 and γ2 mRNA to be more highly expressed in SC than in OM preadipocytes. Therefore, the depot-specific differences in the response of preadipocytes to the proadipogenic effects of the thiazolidinediones may relate to differences in the basal expression levels of both isoforms of PPARγ. The seeming discrepancy with our previous report (21) may represent a real difference between mRNA and protein expression levels. However, Western blotting of PPARγ protein in human preadipocytes is not a highly quantitative method and was performed on a limited number of participants. Furthermore, previous studies indicate that changes in PPARγ mRNA tend to be paralleled by those in protein expression (24–27).

In some biological systems, activation of nuclear hormone receptors increases the expression of the cognate receptor providing a positive feed-forward mechanism (28,29). In our hands, thiazolidinedione treatment of preadipocytes produced a small increase in PPARγ1, which was comparable in OM and SC preadipocytes. Such treatment produced a much more marked increase in PPARγ2 expression. These findings are consistent with those recently reported by Saladin et al. (30), who suggested that differential expression of the two PPARγ isoforms might relate to varying effects of the transcription factor C/EBPβ on the PPARγ1 versus γ2 promoters. In addition to the increased basal levels of PPARγ isoforms in SC preadipocytes, these cells also increase their expression of the γ2 isoform to a higher level compared with OM preadipocytes during differentiation. Because these experiments are performed on primary cultures that have been subcultured, one possible explanation for these findings is that the OM stromovascular cultures could contain a higher proportion of nonadipose cell types compared with those from the SC depot. The lack of any definitive marker of early preadipocytes makes any formal assessment of the level of this contamination problematic. However, given that the results concerning the increase in PPARγ mRNA levels in the presence of rosiglitazone are represented as fold over untreated control, this explanation is unlikely. Thus, the "auto-induction" process for γ2 at least, seems to be more active in SC preadipocytes.

PPARγ binds to its target sequences (peroxisome proliferator response elements [PPREs]) as a heterodimer with the RXRα (31). The natural ligand for RXRα is 9-cis retinoic acid, and synthetic high-affinity rexinoid agonists for this receptor have been developed. These rexinoid compounds have been shown to increase insulin sensitivity in vivo (22). In this study, we demonstrated for the first time that a rexinoid (LG100268) promotes differentiation of human preadipocytes, albeit to a lesser extent compared with PPARγ agonists. The reason for a decreased response to LG100268 compared with rosiglitazone remains unclear. In mouse models of diabetes, Lenhard et al. (32) showed that LG100268 was less effective than rosiglitazone at decreasing serum triglycerides and nonesterified fatty acids and increasing inscapular brown adipose tissue and body weight. LG100268 could be stabilizing RXR/RXR homodimers thereby sequestering RXR and reducing the number of available PPARγ/RXR heterodimers. Alternatively, RXR- and PPARγ-specific agonists may either recruit different coactivator proteins to the PPARγ'2fRXR heterodimer or the same coactivator with different binding affinities. Thus, when bound to PPRE, RXR ligands seem to induce the binding of the p160 class of coactivators, including SRC-1, whereas a PPARγ ligand induced the binding of DRIP205 (33). In addition, Schulman et al. (34) showed that although both rosiglitazone and LG100268 promote the binding of CBP (CREB-binding protein) to the PPAR/RXR heterodimer in a modified mammalian two-hybrid system, this binding was 2.5-fold higher in the presence of rosiglitazone compared with LG100268. Nevertheless, the extent of differentiation in the presence of LG100268 was site-specific with SC preadipocytes being more responsive compared with OM preadipocytes. Therefore, the site-specific responses in human preadipocytes may not be limited to activators of the PPARγ pathway but also include those acting through RXR. In agreement with this observation was the finding that RXRα mRNA expression was significantly higher in SC preadipocytes in the undifferentiated state compared with those derived from the OM depot.

Notably, treatment of preadipocytes with both LG100268 and rosiglitazone resulted in a synergistic increase in preadipocyte differentiation. In agreement, Tontonoz et al. (35) showed that liposarcoma cell differentiation, characterized by an increase in intracellular lipid accumulation, is enhanced by both rosiglitazone and LG100268 and that simultaneous treatment with both ligands results in an additive stimulation. In mouse models of obesity and type 2 diabetes, antidiabetic activity was enhanced by combination treatment with LG100268 and rosiglitazone (22,32). The cooperative effects of PPARγ- and RXR-specific ligands may occur at the level of selective coactivator recruitment.

In summary, both PPARγ1 and -γ2 are more highly expressed in undifferentiated SC, compared with OM, preadipocytes. This may explain, at least in part, why the former are more responsive to the prodifferentiating effects of rosiglitazone. During human preadipocyte differentiation, there is a much greater increase in PPARγ2 than PPARγ1, and this increase is also greater in SC than in OM cells. Additional study of the differential roles of these isoforms is required. RXRα mRNA is also more highly expressed in SC compared with OM preadipocytes. In addition, an RXRα agonist can stimulate human preadipocyte differentiation in a site-specific manner, albeit to a lesser extent than rosiglitazone. The synergistic effects of LG100268 and rosiglitazone suggest that both members of the PPARγ/RXRα heterodimer can contribute to adipogenesis. These data add to our understanding of the regional differences in the response of human preadipocytes to thiazolidinediones, provide novel information regarding the effects of rexinoids on human preadipocyte differentiation, and add to the growing body of evidence supporting

the existence of fundamental regional differences in human adipose tissue biology.

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