Necdin and E2F4 Are Modulated by Rosiglitazone Therapy in Diabetic Human Adipose and Muscle Tissue

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To identify novel pathways mediating molecular mechanisms of thiazolidinediones (TZDs) in humans, we assessed gene expression in adipose and muscle tissue from six subjects with type 2 diabetes before and after 8 weeks of treatment with rosiglitazone. mRNA was analyzed using Total Gene Expression Analysis (TOGA), an automated restriction-based cDNA display method with quantitative analysis of PCR products. The expression of cell cycle regulatory transcription factors E2F4 and the MAGE protein necdin were similarly altered in all subjects after rosiglitazone treatment. E2F4 expression was decreased by 10-fold in muscle and 2.5-fold in adipose tissue; necdin was identified in adipose tissue only and increased 1.8-fold after TZD treatment. To determine whether changes were related to an effect of the drug or adipogenesis, we evaluated the impact of rosiglitazone and differentiation independently in 3T3-L1 adipocytes. While treatment of differentiated adipocytes with rosiglitazone did not alter E2F4 or necdin, expression of both genes was significantly altered during differentiation. Differentiation was associated with increased cytosolic localization of E2F4. Moreover, necdin overexpression potently inhibited adipocyte differentiation and cell cycle progression. These data suggest that changes in necdin and E2F4 expression after rosiglitazone exposure in humans are associated with altered adipocyte differentiation and may contribute to improved insulin sensitivity in humans treated with TZDs. Diabetes 55: 640–650, 2006

Original Article

Thiazolidinediones (TZDs) effectively treat insulin resistance and type 2 diabetes. TZDs bind to peroxisome proliferator–activated receptor-γ (PPAR-γ) nuclear receptors that form heterodimers with retinoid X receptors and complex with coactivators and corepressors to regulate transcription (1). However, the specific mechanisms by which and the tissue sites at which TZDs improve whole-body insulin sensitivity remain incompletely understood (2,3).

PPAR-γ is highly expressed in adipocytes (4), but its expression is less abundant in muscle and liver despite TZDs having significant metabolic effects in these tissues. Thus its potential mechanisms of action include 1) common molecular pathways in muscle and adipose requiring only low levels of PPAR-γ; 2) altered adipocyte secretion of metabolites and adipokines, including free fatty acids, leptin, resistin, and adiponectin, with secondary effects in other tissues (5,6); 3) increased lipid flux into fat and away from muscle (7,8); 4) altered adipocyte differentiation, resulting in increased numbers of small, insulin-sensitive cells (9); 5) changes in the expression of lipid and carbohydrate metabolism genes mediated via PPAR-γ response elements (10,11); or 6) PPAR-γ–independent mechanisms (12).

To better understand the molecular mechanisms by which TZDs act in humans with type 2 diabetes, we sought to identify the genes differentially expressed in human adipose and skeletal muscle in vivo after exposure to the TZD rosiglitazone. We used Total Gene Expression Analysis (TOGA), an automated restriction-based cDNA display technique that offers advantages over other expression analysis methods. First, in contrast to high-density oligonucleotide arrays, this technology allows for the identification and unbiased evaluation of both known and novel mRNAs and splice variants. Second, TOGA is highly sensitive, detecting transcripts expressed at 1 in 106, whereas microarray sensitivity generally ranges from 1 in 103 to 1 in 104 transcripts. Therefore, TOGA analysis requires lower quantities of total RNA (20 ng), making this technique particularly suitable for small human biopsy samples. We have applied this technology for the first time to assess TZD-related responses in humans. We confirmed differential gene expression identified by TOGA using real-time quantitative PCR and performed functional analysis in cultured 3T3-L1 adipocytes.
RESEARCH DESIGN AND METHODS

The Joslin Diabetes Center Human Subject Committee approved the experimental protocol, and informed consent was obtained from all participants. For the study, six subjects with type 2 diabetes not previously treated with a TZD or insulin were recruited. All subjects had normal coagulation and liver function and had no other major systemic illness.

Blood samples were obtained from the subjects in the fasted state for the analysis of glucose, and cholesterol levels and liver function. After subjects were given a local anesthetic with 1% lidocaine, percutaneous biopsies of abdominal subcutaneous adipose tissue and vastus lateralis muscle were performed using a triport cannula and Bergstrom needle, respectively. Samples were immediately frozen in liquid nitrogen. Subjects were then treated with 8 mg/day rosiglitazone (Avandia; GlaxoSmithKline) for 8 weeks, after which the adipose tissue and muscle biopsies were repeated. Compliance with the medication regimen, assessed by pill count, was >94%. (Although techniques are under development to assess TZD drug levels [13], they are not yet widely available for clinical or research use.) We chose the 8-week treatment period because it allowed us to identify gene expression changes that preceded major changes in glycemia, due to potentially confounding effects of glycemic changes on gene expression (14,15).

RNA isolation and TOGA analysis. Tissue samples were homogenized in Trizol (Invitrogen) using a Polytron homogenizer (Brinkmann), with high-salt precipitation modification in muscle and RNase free glycerogen (Ambion) added as a carrier in adipose samples. RNA was similarly isolated from 3T3-L1 cells, with subsequent purification and DNase I treatment over RNeasy columns (Qiagen).

TOGA analysis was performed (16) on mRNA isolated from adipose and muscle samples. cDNA was synthesized using a degenerate pool of biotinylated phosphing primers that initiated synthesis at the poly(A) tail. cDNA was digested with MspI; 3′ fragments were captured using streptavidin beads and released by cleavage with NcoI, which recognized a site in the phasing primers. Captured fragments were modified at their 5′ ends to harbor a start site for T3 polymerase in vitro transcription. RNA fragments were produced corresponding to the 3′ portion of each starting mRNA, from its most 3′ MspI recognition sequence to the beginning of its poly(A) tail, with each fragment flanked by linker tags of known sequence. cDNA was prepared from the RNA pool by reverse transcription and used in four separate PCR s, in which a 5′ primer that extends beyond 20-24 nucleotides beyond the MspI site (N position) was paired with a universal 3′ primer to generate an N-specific double-stranded DNA template. Finally, 256 primers corresponding to all possible permutations of four nucleotides immediately adjacent to the MspI recognition site (N, N, N, and N) were matched with the appropriate N template and used in individual PCRs to produce 256 nonoverlapping product pools that were separated by electrophoresis. This assigned each PCR product an address—eight nucieotides sequence (the four MspI recognition and adjacent four parsing nucleotides) and length, both of which are attributes of individual mRNAs. The fluorescent PCR product peak amplitudes correspond to initial concentrations of parent mRNAs. Data were queried to identify mRNAs whose concentrations differed among experimental samples.

Each sample was analyzed in duplicate, independent reactions. cDNA clones were isolated for interest and sequencing.

Real-time quantitative RT-PCR. Relative levels of differentially expressed genes from tissue and cell culture samples were determined by real-time quantitative RT-PCR (ABI PRISM 7700; Applied Biosystems). cDNA was synthesized using random hexamers (Advantage; Clontech). Primers were selected with Primer Express, and specificity was confirmed by gel (17). PCR was performed using AmpliTaq Gold polymerase, and products were detected with SYBR Green (Applied Biosystems) or FAM- or VIC-labeled probes (target gene and cyclophilin, respectively). (See online appendix Table 1 [available at http://diabetes.diabetesjournals.org] for primer sequences.)

Animal studies. Protocols were approved by the institutional animal care and use committee. ICR mice (Taconic Laboratories) were housed in an Office of Laboratory Animal Welfare–certified facility under standard light/dark cycle conditions. For the experimental group of mice, rosiglitazone was added to Purina 5001 Chow at 3 mg·kg⁻¹·day⁻¹ for 4 months (n = 7 per group).

Cultured cells. Murine 3T3-L1 preadipocytes (American Type Culture Collection) were grown to 90% confluence in Dulbecco’s modified Eagle’s medium with high glucose and 10% calf serum and differentiated with the addition of 5 μg/ml insulin, 0.4 μg/ml dexamethasone, and 0.5 mmol/l isobutylmethylxanthine. Cells were assessed at 2-day intervals from day 0 to day 12 of differentiation. In additional experiments, preadipocytes or day 9 adipocytes were treated with 10 μmol/l rosiglitazone or DMSO for 2 h.

The pRcCMV vector (Invitrogen) subcloned with the 1.6-kb murine necdin fragment was ligated into the EcoRI linearized pBABE retroviral construct. Then 3 μg of necdin-pBABE DNA or empty vector control DNA were transfected into 293 cells (CellPhekt; Pharmaca). The virus was harvested at 48 h by removing the media and passing it through a 45-micron filter. 3T3-L1 cells at 60% confluence were incubated with the virus for 12 h; infected cells were selected using Zeocin (2 μg/ml; Promega). For differentiation studies, equal numbers of stably infected preadipocytes were differentiated in the presence or absence of rosiglitazone (10 μmol/l).

Protein analysis. Triton-soluble and -insoluble cell extracts were separated by SDS-PAGE for Western analysis. Membranes were incubated for 1 h in 3% BSA in PBS/Tween-20 and with primary antibodies for 1 h at room temperature. Antibodies to neccin NC243 (18) and anti-EF2 (Santa Cruz Biotechnology) were used at 1:2,000 and 1:1,000, respectively. Blots were incubated with horseradish peroxidase–conjugated anti-rabbit IgG (Cell Signaling Technology) and visualized using chemiluminescence (PerkinElmer Western Lighting).

Immunochemistry. 3T3-L1 preadipocytes were differentiated on 14-mm cover glasses in 24-well polystyrene plates (Fisher). Cells were washed with PBS, fixed with 1 ml 10% formalin/PBS for 1 h, and permeabilized with 0.1% Triton X/PBS for 5 min. After being washed, cells were blocked with PBS/1.5% goat serum at 4°C overnight, washed again, and incubated with primary antibodies (in PBS/1.5% goat serum) for 1 h. Cells were washed again and then stained with 1:200 fluorescein isothiocyanate–conjugated goat anti-rabbit IgG (Santa Cruz) for 1 h in darkness. Coverslips were mounted with 0.1 mol/l DABCO in 9.1 glycerol/PBS and staining visualized using a confocal microscope at 488 nm.

Triglyceride assays. 3T3-L1 cells were harvested in 0.5 ml PBS. Next 1 ml of 2.1 chloroform/methanol solution was added to the cell pellet and the mixture was vortexed vigorously; 10 μl were then removed for protein analysis. Cells were incubated for 30 min at 4°C, vortexed, and spun at maximum speed for 5 min, after which the supernatant was discarded and methanol/water/chloroform (50:47:3) was added to the pellet. The extract was vortexed and centrifuged for 5 min, and 200 μl of triglyceride reagent (STANBIO) were added. After brief centrifugation, 100 μl of supernatant were removed for spectrophotometry.

Cell cycle analysis. 3T3-L1 cells (cells expressing neccin and control cells) were grown to 90% confluence then synchronized by 24-h starvation. After they were refed for 12 or 24 h in Dulbecco’s modified Eagle’s medium with high glucose and 10% FBS, cells were harvested in PBS/0.1% BSA, washed, and resuspended at 1 × 10⁶ cells/ml. Next 3 ml of cold absolute ethanol were added to 1 ml of cell solution, after which 1 ml of staining solution (3.8 mmol/l sodium citrate, 50 μg/ml propidium iodide in PBS) and 50 μl of RNase A were added. Samples were then stored at 4°C before Coulter XL-MCL analysis.

Statistical analysis. Data are means ± SE. To evaluate relative fold changes in mRNA expression levels in human tissue, the mean indexes using duplicate analysis data were computed. Means of log-transformed expression data were compared using Student’s t test, assuming the null hypothesis of no change. Other statistical analyses were performed using paired or unpaired two-tailed Student’s t tests, as indicated (StatView, SAS).

RESULTS

Human studies. The subjects (four men and two women; five Caucasian and one African American) had a mean age of 49.5 ± 10.8 years, BMI 26.5 ± 3.5 kg/m², HbA1c, 6.9 ± 1.4%, and diabetes duration 1.9 ± 1.6 years. There was no significant change before and after 2 months of rosiglitazone treatment in weight (80.8 ± 14.5 vs. 83.9 ± 17.9 kg; P = 0.1) or fasting glucose (8.6 ± 2.3 vs. 7.6 ± 1.7 mmol/l; P = 0.1), insulin (91.7 ± 31.9 vs. 75.0 ± 33.3 pmol/l; P = 0.3), or C-peptide (0.9 ± 0.3 vs. 0.7 ± 0.3 nmol/l; P = 0.1) levels; all trends were of the expected direction and statistically significant. There was no change in lipids; total cholesterol was decreased by 2.5% (P = 0.06), and HDL cholesterol was increased by 4.3% (P = 0.04).

TABLE 1

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Adipose tissue

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No single subject appeared as a nonresponder across all metabolic variables.

**Expression analysis.** RNA was isolated from each pre- and postrosiglitazone sample and processed individually for TOGA analysis (16). TOGA detected 16,983 mRNA species in adipose tissue and 18,010 in muscle tissue.

To assess the reproducibility of TOGA, a correlation coefficients matrix was generated from duplicate assays (Matlab, Natick, MA). Pearson's product moment correlation analysis demonstrated high reproducibility of replicates ($r^2 > 0.97$) within adipose and muscle tissue. Despite the different sex and ethnic backgrounds of the subjects, the expression of most RNA species within adipose and muscle tissue did not significantly differ among subjects, as was demonstrated by the between-subject similarity scores ($r^2 = 0.84–0.98$).

In four of six subjects, more peaks were altered in adipose than in muscle tissue, as might be predicted from the higher abundance of PPAR-γ in adipose tissue (Table 1). In all, 74 expression peaks differed by $>1.5$-fold in two or more subjects in muscle and/or adipose tissue; only two were concordantly regulated in all six subjects, and these were thus selected for identification and functional evaluation.

The expression of one mRNA was decreased in all subjects after rosiglitazone in both muscle and fat tissue, with a mean reduction in muscle of 81.9% ($P = 0.001$) and in adipose tissue of 49.10% ($P = 0.008$) (Fig. 1A–C).

Sequence of the cloned PCR product identified this mRNA as transcription factor E2F4 (Genbank U15641). Decreased E2F4 mRNA expression was confirmed by real-time PCR in five of six subjects (due to limited tissue in one subject).

A second RNA species increased after rosiglitazone in adipose tissue of all subjects by $1.8 \pm 0.2$-fold ($P = 0.008$).
Expression was low in muscle, with no net change after rosiglitazone ((Fig. 2). The product was identified as necdin (GenBank XM_007686.1). Expression differences were validated in adipose from two subjects due to limited mRNA availability. Thus we also analyzed necdin expression in mouse epididymal fat. Treatment of mice with rosiglitazone (3 mg·kg⁻¹·day⁻¹) for 4 months increased necdin expression by 36% compared with controls (P < 0.003; data not shown).

Analysis in cultured adipocytes. The observed differential expression of E2F4 and necdin in humans after rosiglitazone treatment could be a direct result of the drug and/or PPAR-γ-regulated gene expression or an indirect result of alterations in the differentiation state or systemic changes in metabolic control or insulin sensitivity. To test the impact of rosiglitazone in the absence of complicating systemic metabolism, we evaluated the expression of E2F4 and necdin as a function of differentiation or drug treatment in 3T3-L1 adipocytes.

Expression of E2F4 mRNA decreased significantly by day 2 of differentiation and remained low thereafter (75% reduction at day 2, P < 0.006; 56% at day 12, P = 0.03) (Fig. 3A). E2F4 protein expression in Triton-insoluble nuclear fractions also decreased with differentiation (92% reduction at day 2; P < 0.001) (Fig. 3B). Although the decrease in E2F4 protein expression was not prominent at day 6, it remained low thereafter (91% decrease at day 12; P < 0.001). We also observed the transient appearance of an
additional 130-kDa band at day 2 that was not evident in subsequent days (data not shown); this could have represented nuclear translocation of a related E2F4-immunoreactive protein. In Triton-soluble cytosolic extracts, E2F4 expression increased during early differentiation (69% increase, days 0–4; \( P < 0.05 \)) but declined by late differentiation (66% decrease, days 4–12; \( P < 0.01 \)).

Because alterations in subcellular localization of E2F4 play an important role in cell differentiation (19), we evaluated E2F4 expression using immunocytochemistry (Fig. 3C). By day 2 of differentiation, nuclear E2F4 expression was reduced with relative preservation of cytosolic expression, thus suggesting cytosolic translocation. As differentiation progressed, expression decreased further in the nucleus and cytosol.

To determine whether E2F4 was directly regulated by rosiglitazone, we treated preadipocytes and fully differentiated (day 9) adipocytes with rosiglitazone for 72 h. Although rosiglitazone did not alter mRNA expression of E2F4 in fully differentiated adipocytes, there was a small reduction in preadipocytes (7% decrease, \( P = 0.04 \); data not shown), perhaps related to the limited induction of differentiation with rosiglitazone alone. Similarly, the E2F4 protein level was modestly reduced in rosiglitazone-treated preadipocytes (18% decrease in Triton-insoluble fraction, \( P = 0.03 \); 14% decrease in Triton-soluble fraction, \( P = 0.06 \)) (Fig. 4 and data not shown, respectively) but was not altered by rosiglitazone in fully differentiated cells. In contrast, E2F4 protein expression was markedly reduced as a function of the differentiation state alone (96% decrease; \( P < 0.001 \)) (Fig. 4). These findings suggest a predominant role for the differentiation state rather than a direct effect of rosiglitazone in regulating E2F4 expression in adipocytes.

Like E2F proteins, necdin has been implicated in the regulation of cell cycle progression (20) and differentiation in cultured neuroblastoma cells (21). Therefore, we evaluated the effects of differentiation on necdin expression in 3T3-L1 adipocytes. Differentiation was associated with progressively increased expression of necdin mRNA (40% increase at day 12; \( P = 0.01 \)) (Fig. 5A). Necdin protein levels increased throughout differentiation in both Triton-soluble (57-fold increase; \( P < 0.001 \)) (Fig. 5B) and Triton-insoluble (2.3-fold increase, \( P = 0.001 \); data not shown) extracts. Increased expression of necdin was confirmed by immunocytochemistry (Fig. 5C).

We similarly evaluated direct effects of rosiglitazone on necdin expression. In preadipocytes, rosiglitazone increased necdin mRNA expression, albeit less robustly than differentiation-related effects (23%, \( P = 0.03 \); data not shown). Likewise, protein levels in cytosolic extracts increased dramatically with differentiation (14-fold increase; \( P < 0.001 \)) but not with rosiglitazone (Fig. 6A and B).

E2F (22) and necdin (20) play important roles in cell cycle control, and E2F proteins regulate adipocyte differentiation. Necdin increases differentiation in neuroblastoma cells (21) but may play an inhibitory role in brown preadipocytes (23). To determine a potential functional role of necdin in white adipose tissue differentiation, we generated preadipocytes stably overexpressing necdin (17.5-fold increase in mRNA expression and 10-fold increase in protein levels in 3T3-L1/pBABE-necdin compared with 3T3-L1/pBABE cells). These cells did not differentiate after standard differentiation (Fig. 7A, panel b), even in the presence of 10 \( \mu \)mol/l rosiglitazone (Fig. 7A, panel d). In a similar finding, the accumulation of triglycerides during differentiation was decreased in 3T3-L1/pBABE-necdin cells (Fig. 7E). Although necdin overexpression did not affect E2F4 mRNA (data not shown), necdin reduced mRNA expression of both early (PPAR-\( \gamma \) and CAAAT/enhancer-binding protein-\( \alpha \) [CEBP-\( \alpha \)]) and late (adipocyte fatty acid–binding protein) markers of adipocyte differentiation (Fig. 8A). Likewise, necdin overexpression reduced PPAR-\( \gamma \) protein levels in both cytosolic and nuclear fractions during early differentiation (data not shown). Given the potent inhibition of differentiation in necdin-expressing cells, we assessed the expression of preadipocyte factor 1 (Pref-1 [delta-like 1 homolog]), an important and potent inhibitor of adipocyte differentiation whose expression is normally reduced early after differentiation induction (24). Expression of Pref-1 increased 2.6-fold in 3T3-L1/pBABE-necdin cells (Fig. 8A) and remained substantially higher during differentiation in necdin-expressing cells, even in the presence of rosiglitazone.

Because necdin affects cell cycle progression in neuroblastoma cells (21), we analyzed the impact of necdin on cell cycle progression in 3T3-L1 preadipocytes. Necdin overexpression increased the percentage of cells in G1 (1.8-fold increase at 12 h, \( P < 0.001 \) vs. controls; 1.4-fold increase at 48 h, \( P < 0.001 \) vs. controls) and decreased the percentage of cells in the G2/M (3.3-fold decrease at 12 h, \( P < 0.001 \); 1.5-fold decrease at 24 h, \( P < 0.001 \)) and S (2.7-fold decrease at 12 h, \( P = 0.002 \); 1.2-fold decrease at 24 h, \( P = 0.15 \)) phases (Fig. 8B).

DISCUSSION

Despite the widespread use of TZDs in the treatment of type 2 diabetes, the cellular and molecular mechanisms by which these compounds improve insulin sensitivity remain incompletely understood. To identify genes that have altered expression in vivo in response to rosiglitazone and thus are of potential importance in the pathogenesis of insulin resistance and diabetes, we used the novel TOGA methodology to analyze the expression of both known and
unidentified mRNA in human muscle and adipose tissue. Although studies of gene expression as a function of TZD in humans are ongoing (25–27), we are the first to report on the global differential expression profiles in these tissues from humans exposed to a TZD.

Despite the inherent genetic variability in the human subjects in this study, the expression of most RNA species within adipose tissue and muscle did not differ significantly among the subjects, as demonstrated by their high similarity scores. Overall, rosiglitazone-induced expression changes were small and varied substantially among subjects. The expression of only 74 genes was altered by >1.5-fold in muscle or adipose tissue in at least two subjects after 8 weeks of rosiglitazone. Both the number and magnitude of changes in expression were substantially less than those seen in microarray studies of cells or

FIG. 5. Necdin mRNA expression increases with differentiation in 3T3-L1 cells. A: Necdin expression as a function of differentiation day. *P < 0.05 vs. day 0 levels; **P < 0.01 vs. day 0. B: Representative anti-necdin Western blot of cytosolic extracts (upper panel) and quantification of densitometry results (lower panel). *P < 0.05 vs. day 0; ***P < 0.001 vs. day 0. C: Immunoreactive necdin expression in day (D) 0 and 6 adipocytes.
rodents treated with TZDs (28,29). Several technical and methodological factors might account for these differences: 1) the detection of only mRNA containing *Msp* I restriction sites in our TOGA analysis, 2) between-study differences in drug dosage or duration of exposure, 3) greater intersubject genetic and environmental variability inherent to human studies, and 4) our stringent criteria for gene selection (requiring consistent per-gene expression changes in all subjects). In addition, it is interesting that despite potent changes in insulin sensitivity induced by TZDs, we and others (26,27) do not find insulin-signaling proteins to be highly regulated after TZD exposure.

We focused our analysis on the two genes for which concordant differential regulation has been observed in all human subjects as a function of TZD: E2F4 and *necdin*. Although these two RNA species are unrelated members of the E2F and MAGE families, both are important regulators of cell proliferation, differentiation, and postmitotic survival. Rosiglitazone markedly suppressed E2F4 expression in muscle and adipose tissue but increased *necdin* expression in adipose tissue only. We observed similar effects of differentiation on increasing *necdin* and decreasing *E2F4* expression in 3T3-L1 adipocytes.

**Potential role of E2F4 in TZD effects.** E2F family genes are widely expressed in mammalian tissues and are crucial regulators of the cell cycle. E2F1–3 are inactive when bound to pocket proteins (p107 and p130) during the G1 phase. During the G1/S transition, cyclin-dependent phosphorylation of Rb or pocket proteins results in the release of E2F proteins to be highly regulated after TZD exposure.

Rosiglitazone markedly reduced E2F4 expression in 3T3-L1 adipocytes. Thus, in vivo changes in E2F4 expression are much greater with differentiation than with rosiglitazone; this may reflect the relatively modest ability of rosiglitazone to independently induce differentiation in 3T3-L1 preadipocytes. Thus, in vivo changes in E2F4 expression may be due to direct actions of rosiglitazone on E2F4 expression or be related to prodifferentiation effects of rosiglitazone.

**Potential role of *necdin* in TZD effects.** The second mRNA species altered after rosiglitazone in adipose tissue in vivo was *necdin*, a member of the type II subfamily of the large MAGE family of proteins (35). Many type II MAGE family members are widely expressed in mammalian tissues (18) and cancers (36) and are involved in cellular transformation, growth, and/or apoptosis (35). *Necdin* was initially identified as a nuclear protein expressed in muscle and adipose tissue but increased *necdin* expression in adipose tissue only. We observed similar effects of differentiation on increasing *necdin* and decreasing *E2F4* expression in 3T3-L1 adipocytes.

*Necdin* was initially identified by subtraction cloning in embryonic carcinoma cells induced to neuronal differentiation (37); necdin expression increased markedly during early differentiation. Overexpression of necdin in neuroblastoma cells both suppresses cell growth.
through inhibition of S phase entry and cell cycle arrest (20,47) and induces differentiation (21).

The effect of necdin on differentiation may be specific to cell type. In contrast to neuronal cells, where growth arrest is required before final differentiation, adipocyte differentiation occurs with initial growth arrest, cell cycle re-entry, and clonal expansion and then a second growth arrest followed by terminal differentiation (48). Tseng et al. (23) recently demonstrated increased expression of necdin in insulin receptor substrate 1–deficient brown preadipocytes, which are incapable of differentiation. Reduction of necdin expression (by siRNA or reconstitution of insulin receptor substrate 1) restored the differentiation capacity, indicating an inhibitory effect of necdin on differentiation. These inhibitory effects of necdin were accompanied by reductions in E2F4-stimulated PPAR-γ promoter activity (23). siRNA-mediated reductions in necdin expression also decreased expression of Pref-1, an inhibitor of differentiation.

Our data suggest the importance of necdin in the differentiation of white adipose tissue, a key tissue that is much more abundant than brown adipose tissue in adult humans and that is critical for the maintenance of metabolic homeostasis. Stable expression of necdin in 3T3-L1 adipocytes reduced expression of prodifferentiation regulators CEBP-α and PPAR-γ, reduced lipid accumulation, and markedly increased expression of Pref-1, all consistent with an inhibitory effect of necdin on white adipose differentiation. Although adipogenesis is usually tightly linked to cell cycle control, additional transcriptional effects or pathways may also contribute to necdin inhibition of adipogenesis (34). For example, functional similarity between necdin and Rb also suggests a potential role for interaction between necdin and CEBP family members that is critical for adipocyte differentiation (49). Necdin-overexpressing cells treated with rosiglitazone displayed similar patterns of CEBP-α and PPAR-γ expression, as did untreated control cells. In contrast to control cells, necdin-overexpressing cells were still unable to differentiate, even in the presence of rosiglitazone. These data suggest that additional factors, including increased Pref-1 expression, may contribute to necdin inhibition of adipogenesis.

In addition, we demonstrated that necdin can directly regulate cell cycle progression in white adipocytes. The overexpression of necdin inhibits cell cycle progression, as indicated by increased numbers of cells in the G1 phase and decreased numbers in G2/M and S phases. Inhibition of S phase entry by necdin overexpression has previously

FIG. 7. A: Effect of necdin overexpression in 3T3-L1 cells on differentiation. Shown is a representative field in control (3T3-L1/pBABE) and necdin-overexpressing (3T3-L1/pBABE-Necdin) cells at day 10 that were treated with the standard protocol with or without 10 μmol/l rosiglitazone. The conditions were as follows: control (a), necdin-overexpressing cells (b), control plus rosiglitazone (c), and necdin-overexpressing cells plus rosiglitazone (d). B: Triacylglyceride (TAG) accumulation measured in 3T3-L1 necdin-overexpressing and control cells during differentiation, in the presence of rosiglitazone throughout differentiation. Results are means of two samples and representative of two experiments. ■, necdin-overexpressing cells; □, control.
been demonstrated in stably transfected NIH-3T3 cells and N1E-115 cells, resulting in cell cycle arrest (20,21,47). However, in our studies, stable overexpression of necdin inhibited both cell cycle progression and differentiation of 3T3-L1 preadipocytes, thereby suggesting that 1) necdin effects on differentiation are independent of direct cell cycle regulation and are perhaps mediated via the E2F4 and transcriptional interactions described above or 2) necdin overexpression in preadipocytes also inhibits the initial growth arrest and mitotic clonal expansion required for terminal differentiation.

**Potential coordinate roles of necdin and E2Fs in adipocyte differentiation.** Necdin and E2F4 play prominent, but opposing, roles in the regulation of cell cycle control and differentiation. Thus the identification of increased necdin expression and decreased E2F4 expression by rosiglitazone in humans suggests that a major effect of rosiglitazone may be mediated via effects on cell cycle control and/or adipogenesis. Furthermore, complex reciprocal interactions between these two proteins may regulate transcriptional events (21,23). For example, necdin represses E2F4- and E2F1-mediated transcription (23,47). Conversely, overexpression of E2F4 or E2F1 may antagonize effects of necdin on growth suppression and differentiation (21,23). Although necdin can act as a direct transcriptional repressor via binding to specific guanosine-rich DNA sequences (GN boxes) (50), necdin may also regulate transcription by sequestering E2Fs from target nuclear sites.

Our data in 3T3-L1 adipocytes support a model by which rosiglitazone, together with other prodifferentiation factors, rapidly decreases expression and nuclear localization of E2F4, thereby reducing E2F4 inhibition of cell cycle entry and stimulating early postinduction mitosis and differentiation initiation. At the same time, decreased E2F4 expression/function may derepress necdin, which, in turn,
may induce growth arrest and limit further differentiation. Taken together, the effects of rosiglitazone to decrease E2F4 and increase necdin expression in humans likely reflects TZD-induced enhancement of differentiation (9). We speculate that the effects on necdin and E2F4 expression suggest one mechanism that might limit adipose accumulation with continued TZD exposure.

In summary, our study identified two genes regulated by TZD therapy in humans; we determined that in vivo rosiglitazone exposure decreases E2F4 expression and increases necdin expression. Although both rosiglitazone and the induction of differentiation decreased E2F4 and increased necdin expression, the differentiation state had a greater effect than did TZD exposure, suggesting that changes in human tissue after rosiglitazone exposure are indirect. These data support the concept that one primary mechanism by which TZD therapy acts is via effects on cell cycle control and adipocyte differentiation, resulting in increased numbers of small, well-differentiated adipocytes that may be more insulin sensitive (9). Moreover, direct modulation of these protein families may provide new opportunities for the treatment of insulin resistance and type 2 diabetes.

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