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Regulation of PAI-1 Gene Expression During Adipogenesis

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Abstract Obesity is characterized by elevated levels of circulating plasminogen activator inhibitor-1 (PAI-1), which contribute towards the development of secondary disorders such as type 2 diabetes mellitus and cardiovascular complications. This increase in plasma PAI-1 levels is attributed to an increase in PAI-1 derived from adipose tissue. This study shows that adipose tissue evolved into a major PAI-1 producing organ by gaining capacity during adipocyte differentiation to respond to inducers of PAI-1 transcription. This is mediated by a decrease in E2F1 protein levels, an increase in pRB levels and a decrease in pRB phosphorylation, all leading to a decrease in levels of free E2F, a known transcriptional repressor of PAI-1. Depletion of E2F1–3 was sufficient for inducers such as insulin to potently induce PAI-1 gene expression in pre-adipocytes. Conversely, forced release of pRB-bound endogenous E2F using cell-penetrating peptides can suppress PAI-1 gene expression in adipocytes. This study describes the novel paradigm of cellular differentiation-associated increase in PAI-1 gene expression which is mediated by a decrease in repressor activity, and describes a way of desensitising terminally differentiated cells to PAI-1 inducing agents by restoring endogenous repressor activity. J. Cell. Biochem. 101: 369–380, 2007.

Key words: adipocyte; cardiovascular; insulin

Obesity has reached epidemic proportions globally, with more than 1 billion adults overweight—at least 300 million of them clinically obese—and is a major contributor to the global burden of chronic disease and disability. It is not obesity per se, but its chronic, life-threatening secondary disorders such as type 2 diabetes mellitus (T2DM), cardiovascular complications [Kopelman, 2000] and certain types of cancers [Calle and Kaaks, 2004] that burden healthcare systems worldwide. The precise molecular mechanisms that trigger these secondary disorders under obese conditions have been a subject of intense investigation, but remain inconclusive.

A simplified approach in this direction would be to identify proteins whose levels or activity are altered in obesity and are known independently to contribute to these secondary disorders. Plasminogen activator inhibitor-1 (PAI-1), a physiological inhibitor of plasminogen activators (uPA/tPA) and vitronectin, could be a viable candidate in this regard. Plasma PAI-1 levels are known to be elevated in obese patients [McGill et al., 1994]. Congruently, a dramatic increase in plasma PAI-1 levels (~five-fold) has been observed in genetically obese (ob/ob) mice, in comparison with their lean counterparts [Samad and Loskutoff, 1996]. Conversely, weight loss has led to a subsequent reduction in plasma PAI-1 levels in obese patients [Nielsen and Jensen, 1997; Estelles et al., 2001].

Elevated levels of PAI-1 are implicated in many chronic disorders that are secondary to obesity. PAI-1 is known to play important roles in eliciting thrombosis, fibrosis and vascular remodeling, thus contributing to a wide variety of cardiovascular disorders. The role of PAI-1 in cardiovascular complications has been reviewed elsewhere [De Taeye et al., 2005].

It has been shown that PAI-1 can inhibit the interaction between vitronectin and $\alpha_v\beta_3$

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integrin [Stefansson and Lawrence, 1996]. The disruption of this interaction by PAI-1 was shown to cause insulin resistance [Lopez-Alemany et al., 2003]. In-line with these observations, it has recently been show that PAI-1 knock-out mice are resistant to high-fat-diet-induced insulin resistance [Schafer et al., 2001; Ma et al., 2004], indicating a role for PAI-1 in the pathogenesis of obesity-driven insulin resistance and hence T2DM. Moreover, levels of plasma PAI-1 have been shown to be a prognostic marker of T2DM independent of insulin resistance and other known risk factors for diabetes [Festa et al., 2002].

The role of PAI-1 in cancer progression is rather controversial. Nevertheless, in almost all cancer types that are associated with obesity [Calle and Kaaks, 2004], a role for PAI-1 has been proposed. Possible mechanisms by which PAI-1 contributes to cancer dissemination include the prevention of excess degradation of the extra-cellular matrix, modulating cell adhesion [Palmieri et al., 2002], promoting angiogenesis [Bajou et al., 2001], inhibiting apoptosis [Kwaan et al., 2000] and stimulating cell proliferation [Webb et al., 2001]. Taken together, elevated levels of PAI-1 in obese patients may be an important factor contributing to obesity-driven chronic secondary disorders. Needless to say, understanding the molecular mechanisms that lead to PAI-1 upregulation in obesity assumes paramount importance.

The increase in plasma PAI-1 levels that is associated with obesity could be attributed to adipose tissue. In addition to adipocytes, adipose tissue contains stromal-vascular cells including fibroblastic connective tissue cells, leukocytes, macrophages, and pre-adipocytes. There is an ongoing debate if adipocytes or stromal cells are the major PAI-1 producing components within adipose tissue [Samad and Loskutoff, 1996; Bastelica et al., 2002]. Adipose tissue is one of the largest endocrine organs in obese patients [Dellas and Loskutoff, 2005]. Weight loss in humans, either by exercise [Folsom et al., 1993] or by surgical removal of fat [Primrose et al., 1992], leads to a decrease in plasma PAI-1 levels, which rise again if weight is regained [Mavri et al., 1999]. Furthermore, comparative analysis of PAI-1 gene expression in various tissues of ob/ob mice and their wildtype counterparts shows that, in ob/ob mice, PAI-1 upregulation was significantly higher in the adipose tissue but only modestly increased in other tissues. These observations indicate that adipose tissue is a major, possibly the largest, contributor to plasma PAI-1 in conditions of obesity [Samad and Loskutoff, 1996].

Adipose tissue is primarily composed of adipocytes that are formed by the differentiation of pre-adipocytes, a process commonly referred to as adipogenesis. Under serum-starved conditions, neither 3T3L1 pre-adipocytes nor differentiated adipocytes showed any significant PAI-1 gene expression (see Results). But, in the presence of serum (see Results) or insulin [Seki et al., 2001], adipocytes, but not pre-adipocytes, showed significant PAI-1 gene expression. Taken together, this suggests that adipogenesis, as such, may not induce PAI-1; rather it enhances the potential of cells to respond to PAI-1 inducers (e.g., insulin) in serum.

Hyperinsulinemia has been shown to be associated with obesity and insulin resistance. The evidence for the pathophysiological role of insulin in the elevation of PAI-1 comes from the following observations. Conditions that increase endogenous plasma insulin levels (e.g., high calorie, high carbohydrate meals) were associated with increases in plasma PAI-1, whereas conditions that reduced endogenous insulin (e.g., fasting or treatment with metformin or troglitazone) were associated with decreases in plasma PAI-1 [Juhan-Vague and Alessi, 1997; Kruszynska et al., 2000]. Moreover, administration of exogenous insulin in rabbits [Nordt et al., 1995], mice [Samad and Loskutoff, 1996], and human subjects [Carmassi et al., 1999] significantly increased plasma PAI-1 levels.

The E2F family of transcription factors are known to reversibly bind to the pocket proteins (pRB, p107, and p130) and are key regulators of the cell cycle. E2F1–3 complex with pRB, and the hyperphosphorylation of pRB leads to the release of E2F1–3 isoforms. Contrary to the earlier belief that E2F1–3 are transcriptional activators and E2F4–5 are transcriptional repressors [Trimarchi and Lees, 2002], we have previously demonstrated that free E2F1–3 (unbound to pocket proteins) act as transcriptional repressors of the PAI-1 gene in a cell type-independent manner [Koziczak et al., 2000, 2001]. Using chromatin immunoprecipitation assays, it was also shown by other laboratories

that E2F1–3 can directly bind to the PAI-1 promoter in a tissue specific-manner [Wells et al., 2002]. Subsequently, other groups have shown repressive effects of E2F1 [Croxton et al., 2002], E2F2 [Murga et al., 2001], and E2F3 [Aslanian et al., 2004] on other target genes. As the compromise of E2F activity was shown to be required for cellular differentiation [Muller et al., 2001], it would be intriguing to investigate if it can also be held responsible for the enhanced PAI-1 biosynthetic potential attained during adipogenesis.

As adipose tissue is the major contributor of plasma PAI-1, the crux of obesity-associated increase in plasma PAI-1 levels lies in the enhanced PAI-1 biosynthetic potential acquired during adipogenesis. It is this gain of function that enables the adipocytes to respond to various stimuli to express and secrete PAI-1. In this study, we investigated the mechanisms by which adipogenesis empowers the cells to respond to PAI-1 inducers such as insulin, which happens to be a major physiological stimulus in conditions of diabetes and obesity. We also explored the potential of inhibiting PAI-1 gene expression in terminally differentiated adipocytes by the forced release of endogenous E2F (bound to pRB) without effectuating cell-cycle re-entry.

MATERIALS AND METHODS

Reagents

Insulin (I-5500) was obtained from Sigma. Monoclonal antibodies against E2F1 (KH-95) and E2F2 (TFE-25), and rabbit polyclonal antibodies against E2F3 (C-18), E2F4 (C-20), E2F5 (C-20), pRB (M-153), p130 (C-20), p107 (C-18), IRS-1 (C-20), Erk, and PAI-1 (H-135) were from Santa Cruz Biotechnology. Rabbit polyclonal antibodies against phospho-pRB (Ser-795) and phospho-Erk were from Cell Signaling. Rabbit polyclonal antibodies against Shc and phospho-tyrosine (G410) were from Transduction Laboratories. Sheep polyclonal antibody against PAI-1 was from American Diagnostics. 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole (DRB) was from Fluka. BrdU incorporation kit (Cat. no. 1296736) was from Roche Diagnostics.

Adipocyte Differentiation

3T3-L1 pre-adipocytes were cultured in DMEM containing 10% FCS, and 2 days after cells reached confluency differentiation was induced by changing the culture medium to DMEM containing 10% FCS, 10 μg/ml insulin, 1 μM dexamethasone, and 0.5 mM isobutylmethylxanthine. Two or 3 days later, this medium was replaced with DMEM supplemented with only 10 μg/ml insulin, and cells were kept for 2 days. The medium was then replaced with DMEM containing 10% FCS every 2 days. Cellular morphology was observed using a Nikon Diaphot inverted microscope (10× objective with numerical aperture of 0.25).

Oil-Red O Staining

Cells were washed twice with phosphate-buffered saline (PBS) and fixed with 3.7% formaldehyde solution for 1 h. After washing twice with PBS, cells were stained with oil-red O [60:40 (v/v) dilution in water of 0.5% stock solution (v/v, in isopropanol)] for 1 h. Cells were then washed twice with PBS and twice with water.

RNA Isolation and Northern Blot Analysis

Total RNA (12 μg) was isolated using acid-guanidinium thiocyanate-phenol-chloroform method and subjected to Northern blot analysis as previously described [Koziczak et al., 2001].

Nuclear Run-on Transcription Assay

Transcriptionally active nuclei from pre-adipocytes and adipocytes were extracted [Venugopal et al., 2004] and the amount of nuclei was equalized based on the 280 nm absorbance of the total nuclear lysate. A run-on assay was performed in the presence of [α-^{32}P]UTP according to the basic protocol [Lagor et al., 2005], with minor modifications. Purified mRNA from both samples was hybridized to nylon filters containing linearized and immobilized PAI-1 and GAPDH cDNA (1 μg of each) and exposed to a phosphoimager.

Quantitative Real-Time PCR

Total RNA extraction for RT-PCR was done using RNeasy kits (Qiagen) according to the instructions from the manufacturer. RT-PCR for PAI-1 was carried out as described [Venugopal et al., 2004].

Immunoprecipitation and Western Blotting

Immunoprecipitation and Western blotting were performed as previously stated [Venugopal et al., 2004].

siRNA Nucleofection

Small interfering RNA (siRNA) used for targeting E2F mRNA has the following sequence: E2F1: sense, 5′-GAC UCC UCG CAG AUC GUC AUC-3′ and antisense, 5′-UGA CGA UCU GCG AGG AGU CGA-3′; E2F2/3: sense, 5′-ACA UCA CCA ACG UGC UGG AAG-3′ and antisense, 5′-UCC AGC ACG UUG GUG AUG UCG-3′; control siRNA: sense, 5′-GUAC-CUGACUAGUCGFCAGAAG-3′ and antisense, 5′-UCU GCG ACU AGU CAG GUA CGG-3′. Each siRNA (final concentration 1 μM) was mixed with 3T3L1 cell suspension (1×10^6 cells in 0.1 ml buffer-V/transfection), transferred to a 2-mm electroporation cuvette, and electroporated using an Amaxa Nucleofector™ (Amaxa, Germany) using program T-20. After electroporation, cells were immediately transferred to 1 ml growth medium, and cultured in six-well plates at 37°C until analysis.

Electromobility Shift Assays

Nuclear extracts (5 μg) were first incubated at room temperature for 15 min in 20 μl binding reaction mixture containing 50 mM KCl, 20 mM HEPES (pH 7.9), 0.2 mM EDTA, 6% glycerol, 0.5% Ficoll 400, 1 μg salmon sperm DNA, 6 μg bovine serum albumin and 1 mM DTT with or without penetrating peptide and antibodies, followed by a further 15-min incubation after addition of 0.3 ng radiolabeled oligonucleotide probes. Oligonucleotide probes were radiolabelled using *E. coli* polynucleotide kinase and [γ-^{32}P]ATP. Aliquots (5 μl) of reaction mixture were separated in a 4.5% polyacrylamide gel run in 0.25× TBE buffer at room temperature. The gel was dried and analyzed in a PhosphorImager.

Cell-Penetrating Peptide Treatment

The sequence of the interfering peptide was derived from the pRB-binding region of E2F1 (aa 402–419: LDYHFGLEEGEGIRDLFD) [Helin et al., 1992]. A control peptide with the same amino acid composition, GEE-LEGFHDGLLDFDIR, was prepared by randomly shuffling the sequence of this peptide. Cell-penetrating peptides were prepared by coupling these peptides to the carboxyl terminal of the cell-penetrating region of the HIV tat protein (aa 47–57: RRRQRRKKR) via hinge peptide G. Differentiated adipocytes were separated from undifferentiated cells using a Percoll density gradient as previously described [Venugopal et al., 2004]. The penetrating peptide was then added to cells, incubated for 16 h and then subjected to various treatments.

BrdU Incorporation Assay

Adipocytes were enriched using a Percol density gradient as described above. About 1×10^5 adipocytes were then seeded in a six-well plate containing cover slips, incubated overnight in growth media and then replaced by bromodeoxyuridine (BrdU)-containing media. The cells were subjected to various treatments for 48 h, washed with PBS, fixed with ethanol fixative (25 mM glycine in 100% ethanol; pH 2) at −20°C for at least 20 min and then subjected to immunofluorescence analysis according to the instruction from the manufacturers (BrdU Detection Kit-1, Roche). Cellular morphology was observed using a Nikon Diaphot inverted microscope (10× objective with numerical aperture of 0.25). Fluorescence was visualized with a Zeiss Axioplan fluroscence microscope (63×oil objective with numerical aperture of 1.4).

RESULTS

PAI-1 Levels Are Upregulated During Adipogenesis

The differentiation of 3T3L1 pre-adipocytes into adipocytes was verified by microscopic observation of cell morphology changes (Fig. 1A) and lipid staining using Oil-Red O (Fig. 1B). Eight days after the induction of differentiation, almost all cells contained vacuoles, a lipid storage compartment, and were positive for lipid staining. The differentiation process (adipogenesis) was accompanied by an increase in PAI-1 gene expression, which was comparable to the increase in the gene expression of PPARγ, a well-known adipocyte differentiation marker (Fig. 1C).

Increase in PAI-1 mRNA Levels Is Transcriptional

The increase in mRNA levels could be due to an increase in either mRNA synthesis, mRNA stability, or both. To investigate this, we looked at the state of PAI-1 gene transcription in both pre-adipocytes and adipocytes by nuclear run-on assays. PAI-1 transcription appears to be limited in pre-adipocytes, while substantial in adipocytes (Fig. 2A). Furthermore, we examined the half-lives of PAI-1 mRNA in pre-adipocytes and adipocytes using DRB chase experiments. As shown in Figure 2B, the

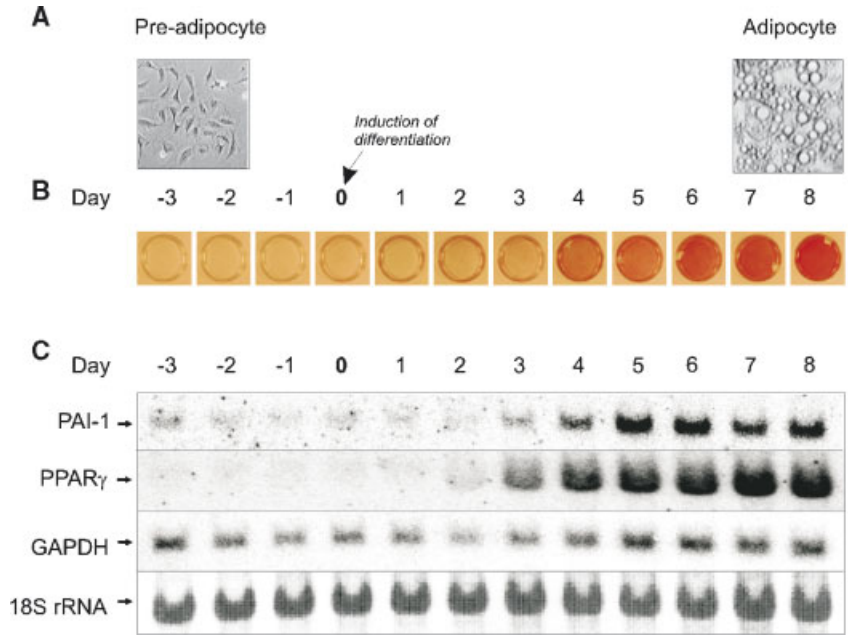

Fig. 1. PAI-1 gene expression during adipogenesis. 3T3L1 cells were differentiated and subjected to (**A**) microscopic examination and (**B**) oil-red O staining. **C**: The total RNA was collected from cells on every day of adipogenesis, and mRNA levels of PAI-1, PPAR-gamma and GAPDH (control) were measured using Northern blot analysis. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

half-lives of PAI-1 mRNA in pre-adipocytes and adipocytes were 4.2 and 1.8 h, respectively. This result indicates that PAI-1 mRNA becomes rather unstable during adipogenesis. Therefore, the elevation of PAI-1 mRNA levels in adipocytes is due to transcriptional activation of the PAI-1 gene and does not involve an increase in mRNA stability.

Increase in PAI-1 Gene Expression Is a Result of Enhanced Biosynthetic Potential Acquired During Adipogenesis

Cellular gene expression can be influenced by both extrinsic (e.g., extracellular growth factors) and intrinsic factors (e.g. transcription factors). To understand the relative role of these factors in the increase of PAI-1 gene expression associated with adipogenesis, the cells were deprived of extracellular inducers by culturing them overnight in the absence of serum. Interestingly, serum-starved cells, irrespective of their state of differentiation, expressed very low levels of PAI-1 mRNA, which did not change during adipogenesis (Fig. 3). Serum starvation did not lead to cell death or apoptosis (data not shown). On the other hand, when supplemented with insulin, these cells could induce PAI-1 gene expression in a manner positively correlating with the extent of differentiation. Even high concentrations of insulin (1.7 μM) could barely induce PAI-1 gene expression in pre-adipocytes, whereas in terminally differentiated adipocytes more than seven-fold induction was observed. These results strongly suggest that adipogenesis as such may not induce PAI-1; rather, it enhances the potential of cells to respond to PAI-1 inducers (e.g., insulin) in serum.

Inducibility of the PAI-1 Gene Is Associated With a Decrease in Free E2F Levels

We have previously shown that free E2F1–3 that is not bound to pocket-proteins can act as a transcriptional repressor of PAI-1 gene expression. Removal of repressor activity is a plausible mechanism through which inducers can gain biosynthetic potential. To find out if an inverse correlation between E2F activity and PAI-1 gene expression during adipogenesis exists, we employed gel-shift assays to assess the state of E2F activity (free E2F) using an E2pro oligonucleotide probe that contains E2F recognition sites derived from the adenovirus E2 promoter [Shirodkar et al., 1992]. Both free and complex forms of E2F1–6 are able to bind to this E2F recognition element, and can be distinguished from each other by differential migration in DNA

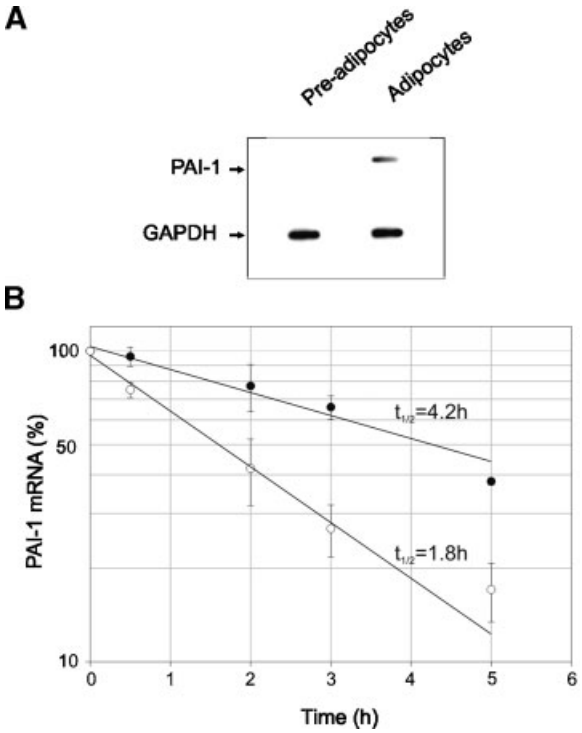

Fig. 2. PAI-1 mRNA stability during adipogenesis. **A**: Transcriptionally active nuclei from both pre-adipocytes and adipocytes were subjected to run-on assays. Purified mRNA from both samples were hybridised to nylon membranes containing linearized and immobilized cDNA from PAI-1 and GAPDH and then exposed to a phosphoimager. **B**: Both pre-adipocytes (filled circles) and adipocytes (open circles) were treated with DRB (20 μg/ml) and cells were collected at various time points. PAI-1 levels normalized to GAPDH levels were plotted on a semi-log scale and linear regression lines drawn using SigmaPlot.

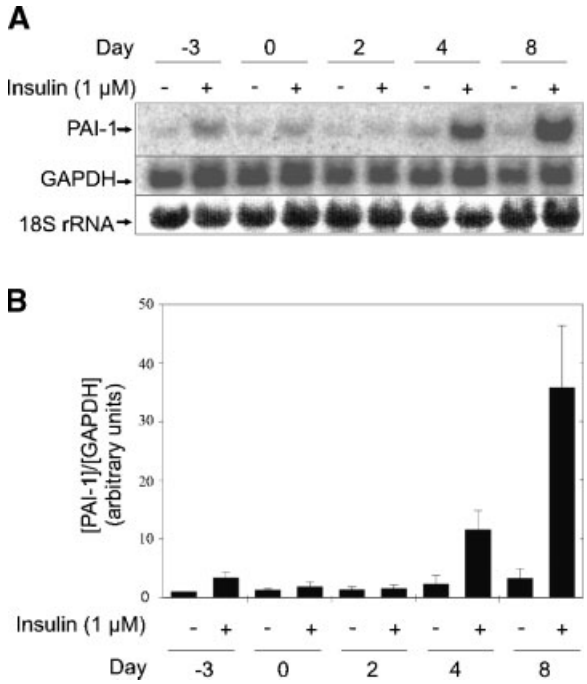

Fig. 3. The role of serum and insulin in effecting PAI-1 gene expression during adipogenesis. **A**: Cells at various stages of adipocyte differentiation were subjected to overnight serum-starvation and then induced with 10 μg/ml insulin (1.7 μM) for 2 h. Total RNA was prepared and analyzed for PAI-1 and GAPDH levels by Northern blot analysis. **B**: PAI-1 gene expression from three independent experiments were quantified and normalized with GAPDH.

gel-shift assays. The pattern of protein-DNA complexes showed differentiation-associated changes in gel-shift assays (Fig. 4A). In proliferating pre-adipocytes (day-3), several sizes of DNA-protein complexes were detected and, upon reaching confluency (day 0), the intensity of a higher band (B) and lower bands (C) declined and a new band (D) appeared. During differentiation (day 0–8), a further decrease in the intensity of the lower bands (C) was observed. The specificities of these DNA-protein complexes were examined by competition experiments using a competitor oligonucleotide with an E2F recognition sequence derived from the dihydrofolate reductase (DHFR) promoter [Herber et al., 1994]. Except for band A, all complexes found in extracts of pre-adipocytes and adipocytes were outcompeted by the DHFR promoter-derived specific oligonucleotide, but not by a mutated oligonucleotide, in a dose-dependent manner (Fig. 4B), suggesting that these bands represents E2F isoforms that are either free or in complexes with other protein(s). Super-shift assays of adipocyte (day 8) nuclear extracts using specific antibodies revealed that complex D contained E2F bound to p130 and complex E contained E2F bound to pRB (Fig. 4C). The fast-migrating DNA-protein complexes (C) are present only in proliferating pre-adipocytes, but are progressively reduced during differentiation. These bands, super-shifted with antibody against E2F1 (Fig. 4D), reflects free, active E2F. Other complexes may represent higher molecular weight forms comprising E2F and other proteins such as p107, HDAC, cdk2, cyclin A, and DP [Richon et al., 1997; Timchenko et al., 1999]. Taken together, a significant reduction in free E2F levels is found to be associated with adipogenesis.

Decrease in Free E2F Levels May be Due to Changes in Protein Levels of E2F1 and pRB, and the Phosphorylation Status of pRB

In principle, the decrease in free E2F levels could be due to a decrease in E2F protein levels, an increase in pRB protein levels or a decrease

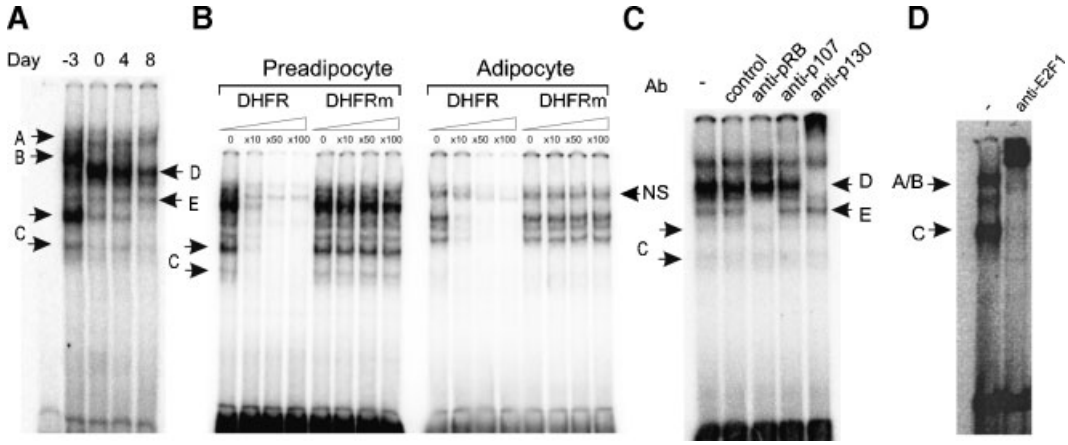

Fig. 4. E2F DNA-binding activity during adipogenesis. **A**: E2F DNA-binding activity of nuclear extracts from various days of adipogenesis was assessed by EMSA. **B**: In the gel-shift assays, nuclear extracts prepared from adipocytes (day 8) were incubated with the same radioactive oligonucleotide used in (A) together with an increasing amount of a cold oligonucleotide DHFR containing an E2F-binding site or mutant DHFRm. **C**: Nuclear extracts from adipocytes (day 8) were preincubated with different antibodies for 15 min and then analyzed for E2F DNA-binding activity by EMSA. **D**: Nuclear extracts (day 2) were pre-incubated with anti-E2F1 antibody for 15 min and analyzed by EMSA.

in pRB phosphorylation. To understand how E2F activity is reduced during adipogenesis, the protein levels of E2F1–3 and pRB, as well as the phosphorylation levels of pRB, were assessed. E2F1 levels were progressively reduced during adipogenesis, while E2F2 and E2F3 levels remained unchanged (Fig. 5A,B). It appears that adipogenesis results in decreasing pRB phosphorylation status (Fig. 5C) and increasing pRB protein levels (Fig. 5D). In this analysis we did not consider the other pocket-proteins, p107 and p130, as they do not bind to E2F1–3.

Increase in PAI-1 Gene Expression Is a Result of a Decrease in E2F Activity

Further to our demonstration of an inverse correlation between E2F activity and PAI-1 levels (Fig. 4), we sought to verify whether this

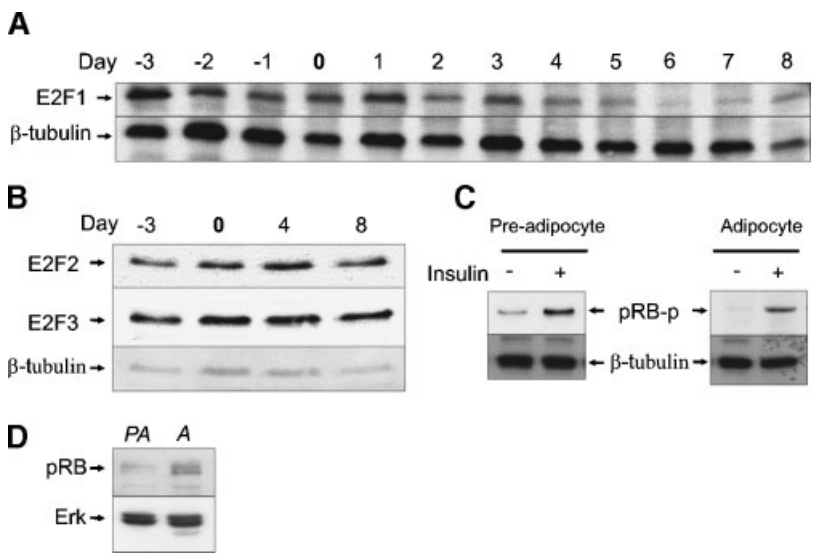

Fig. 5. Analysis of the mechanisms that contribute to a decrease in free E2F levels during adipogenesis. **A**: E2F1 and (**B**) E2F2/3 protein levels were measured at various stages of adipogenesis. **C**: Both pre-adipocytes and adipocytes were treated with or without insulin (100 nM) for 10 min and analyzed for pRB phosphorylation. **D**: 500 μg total cell lysates of pre-adipocytes and adipocytes (day 8) were first immunoprecipitated and then immunoblotted with anti-pRB antibody.

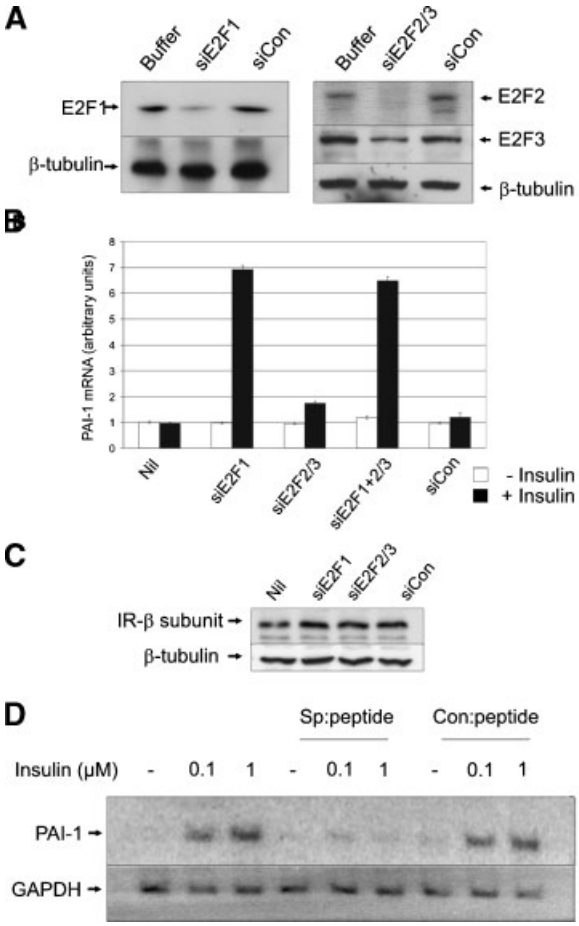

Fig. 6. Effect of E2F1–3 on PAI-1 gene expression in pre-adipocytes and adipocytes. **A**: 3T3L1 pre-adipocytes were electroporated with siRNA against respective E2F isoforms. After 48 h, the cells were collected and analyzed for E2F1–3 isoforms using Western blot analysis. **B**: Cells were electroporated with siRNA against respectiveE2F isoforms. After 32 h, the cells were subjected to serum starvation for about 16 h, followed by treatment with or without insulin (1 μM) for 2 h. The total RNA was extracted from the cells and then analyzed for PAI-1 mRNA levels using RT-PCR with 18S rRNA as an internal control. **C**: Cells treated with siE2F1–3 were analyzed for insulin receptor levels (**D**) Fully differentiated adipocytes were treated with specific or control peptide for 16 h followed by treatment with insulin for 2 h. PAI-1 and GAPDH mRNA levels were analyzed using Northern blot analysis.

correlation is non-consequential or if the decrease in E2F activity is indeed responsible for the increase in PAI-1 levels during adipogenesis. To address this question, we performed RNAi-mediated gene silencing of E2F1–3 in pre-adipocytes by electroporating siRNAs that target E2F mRNA. Downregulation of E2F isoforms was found to be most effective 48 h after electroporation using protocol T-20 (data not shown). As shown in Figure 6A, transfection of cells with specific siRNAs directed against E2F1 and E2F2/3 mRNAs downregulated the respective proteins. Under serum-starved conditions, neither siE2F1, siE2F2/3 nor insulin treatment by themselves had any significant effect on PAI-1 levels, but insulin strongly induced PAI-1 mRNA (Fig. 6B) in cells pretreated with siE2F. siE2F treatment did not alter insulin receptor levels (Fig. 6C).The magnitude of PAI-1 induction was highest upon depletion of E2F1–3 or E2F1 alone followed by E2F2/3. These results indicate that downregulation of E2F1–3 enhances the potential of insulin to induce PAI-1 gene expression in pre-adipocytes.

Conversely, the cell-penetrating peptide that physically disrupts the E2F-pRB binding, and thereby releases E2F isoforms [Venugopal et al., 2004], was found to significantly reduce insulin-induced PAI-1 mRNA levels in differentiated adipocytes (Fig. 6D). This indicates that an increase in free E2F1–3 levels diminishes the potential of insulin to induce PAI-1 gene expression in adipocytes.

Reactivation of E2F by Cell-Penetrating Interfering Peptide Reduces PAI-1 Gene Expression Without Causing Cell-Cycle Re-Entry

The dramatic reduction of insulin-induced PAI-1 levels by the cell-penetrating interfering peptide is supportive of the novel pharmacological paradigm of disrupting E2F-pRB interaction to suppress PAI-1 levels. The main concern in this strategy is that the forced release of free E2F may induce cell-cycle re-entry and uncontrolled cell growth in terminally differentiated cells. We therefore examined the effect of these peptides on DNA synthesis in adipocytes. Terminally differentiated adipocytes were treated with specific (40 μM) and control peptide (40 μM) for 48 h, analyzed by light microscopy and then subjected to BrdU incorporation assay. A mutant of human adenovirus type 5 (dl520) that is known to induce cell-cycle re-entry in adipocytes was used as a positive control [Crescenzi et al., 1995]. Untreated cells and cells treated with specific peptide or control peptide showed similar morphology and absence of BrdU incorporation (Fig. 7), while the dl520-treated cells showed altered morphology and BrdU incorporation. This suggests that interfering peptide treatment does not lead to cell-cycle re-entry in terminally differentiated adipocytes.

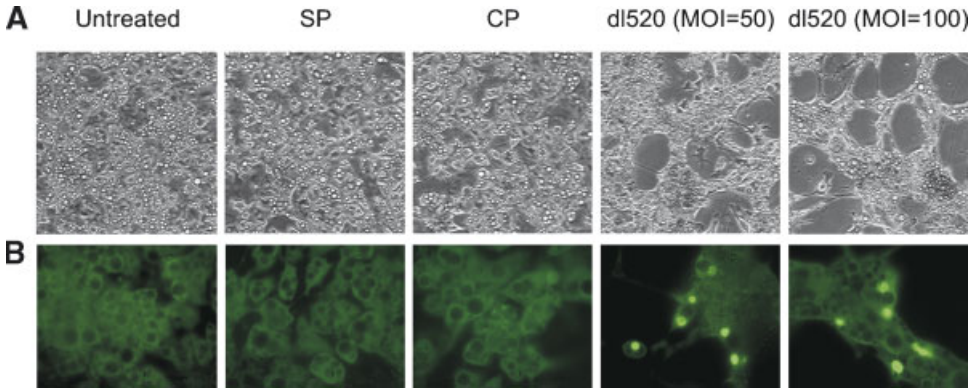

Fig. 7. Effect of cell-penetrating interfering peptide on BrdU incorporation. Differentiated adipocytes were treated either with specific peptide (18 h), control peptide (18 h) or dl520 (48 h). **A**: The cells were analyzed using microscopic examination and subjected to (**B**) BrdU incorporation assay. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

DISCUSSION

Adipose tissue, a major source of plasma PAI-1, is thought to be the largest endocrine organ in obese patients [Hug and Lodish, 2005], thus offering a plausible explanation as to why elevated plasma PAI-1 levels are a biochemical hallmark of obesity. This study was aimed at understanding the molecular mechanisms which enable adipocytes to be a major PAI-1 producing organ and to pharmacologically modulate those mechanisms to inhibit PAI-1 production in adipocytes. In agreement with other studies [Seki et al., 2001], we have shown that PAI-1 gene expression is significantly upregulated during adipogenesis (Fig. 1C). The results of nuclear run-on assay suggest that this increase is transcriptional, while DRB chase experiments rule out a role for mRNA stability (Fig. 2). Interestingly, 3T3L1 cells, irrespective of their state of differentiation, failed to induce PAI-1 under serum-starved conditions (Fig. 3). However, in the presence of serum (Fig. 1C) or insulin (Fig. 3), adipocytes, but not pre-adipocytes, showed significant PAI-1 gene expression, corresponding to the state of differentiation. Taken together, these results indicate that adipogenesis as such does not induce PAI-1; rather, it enhances the potential of cells to respond to PAI-1 inducers (e.g., insulin) in serum.

This study clearly shows an association between the decrease in free E2F levels (Fig. 4A) and the increase in PAI-1 gene expression (Fig. 1C) during adipogenesis. The decrease in free E2F levels is mediated through at least three mechanisms: a decrease in E2F1 protein levels; an increase in pRB levels; and a decrease in pRB phosphorylation (Fig. 5). Previous studies from our laboratory have shown that free E2F1–3 act as transcriptional repressors of PAI-1 [Koziczak et al., 2000]. Therefore, it was intriguing to investigate if reduction of free E2F levels is responsible for the increase in PAI-1 inducibility during adipogenesis.

However, adipocyte differentiation is accompanied by an increase in cellular levels of the insulin receptor [Shimizu et al., 1986] and PPARγ [Chawla et al., 1994], both known to positively regulate PAI-1 gene expression. It was thought that the dramatic increase in PAI-1 gene expression during adipocyte differentiation is a direct consequence of these changes. One could argue that the inverse correlation between free E2F1–3 levels and PAI-1 gene expression can therefore be non-consequential. In pre-adipocytes under serum-starved conditions, neither siE2F1, siE2F2/3, nor insulin treatment by themselves had any significant effect on PAI-1 mRNA levels (Fig. 6B). However, insulin treatment could strongly induce PAI-1 gene expression in cells treated with siE2F1–3. This tells us three things. Firstly, removal of repressor activity alone is not sufficient to induce PAI-1. This requires inducers and that might explain why high plasma PAI-1 levels are often associated with high levels of inducers such as insulin and tumor necrosis factor-α (TNF-α). Secondly, in the absence of E2F1–3, insulin signaling is robust enough to induce PAI-1 in pre-adipocytes. This is surprising as pre-adipocytes are known to have very low expression of the insulin receptor and PPARγ,

but these results are in agreement with the spare receptor status of the insulin receptor. In other words, these results challenge the conventional notion that increases in insulin-induced PAI-1 gene expression during adipogenesis is merely a result of an increase in expression of the insulin receptor. Thirdly, the results also suggest that E2F1 is a stronger repressor than E2F2/3 in pre-adipocytes. It can be argued that the PAI-1 upregulation upon siE2F treatment could be a result of enhanced insulin signaling and not due to removal of repressor activity. We have ruled this out, by showing that the insulin receptor steady state levels remains unchanged after siE2F treatment. Conversely, release of free E2F using an interfering peptide was sufficient to significantly reduce insulin-induced PAI-1 gene expression in adipocytes (Fig. 6D). These results provide strong evidence that, irrespective of the state of differentiation, PAI-1 gene expression during adipogenesis is dependent on free E2F levels. This doubtless confirms that a reduction in free E2F levels, hence the relieving of transcriptional repression, is partly, if not wholly, responsible for the increase in PAI-1 gene expression during adipogenesis. Our findings are further supported by data from E2F1$^{-/-}$ mice that show a 5.7-fold increase in hepatic PAI-1 mRNA levels when compared to their wildtype counterparts [Wells et al., 2002].

Recently, an orally active small molecule antagonist of PAI-1 (PAI-039) was shown to inhibit plasma PAI-1 activity and accelerate fibrinolysis of coronary artery thrombosis in dogs [Hennan et al., 2005] and provide protection against angiotensin II-induced aortic remodeling in mice [Weisberg et al., 2005]. These studies offer the much-awaited proof-of-concept for PAI-1 inhibitors in treating cardiovascular complications, thereby endorsing the concept of PAI-1 inhibitors to treat secondary complications of obesity. Since the reactivation of E2F using cell-penetrating interfering peptide could be successfully employed to reduce PAI-1 levels in terminally differentiated adipocytes (Fig. 6), small molecule antagonists of E2F-pRB interaction may offer therapeutic value. E2F isoforms that bind to pRB (E2F1–3) are transcriptional factors that drive cell proliferation and apoptosis. Therefore, the pharmacological strategy of disrupting E2F-pRB interaction raises a major concern because it may lead to unwanted effects such as cell-cycle re-entry in terminally differentiated adipocytes. For example, adenoviruses harboring E1A could force terminally differentiated Adipo5–2 adipocytes and C2C12 myoblasts to re-enter the cell cycle [Crescenzi et al., 1995]. Although E1A is known to act on multiple targets, including E2F, p300, pRB, p107, p130, TBP, and AP-1, E2F activity was required for E1A-mediated cell-cycle re-entry [Pajalunga et al., 1999]. On the other hand, it has been shown that E2F activation alone, either by overexpressing E2F [Pajalunga et al., 1999] or by downregulating pRB [Huh et al., 2004], does not cause cell-cycle re-entry in terminally differentiated skeletal muscles. In line with these observations, we show that the cell-penetrating peptide that releases free E2F and thereby suppresses PAI-1 gene expression does not cause cell cycle re-entry (Fig. 7).

Although our data linking the decrease in E2F activity to an increase in PAI-1 gene expression are limited to adipocyte differentiation, the results could have wider implications. It has been shown that E2F1–3 regulate many genes that are involved in cell proliferation and differentiation [Muller et al., 2001]. Furthermore, reduction in the activity or levels of E2F isoforms (mainly E2F1) is critical for cell cycle arrest and subsequent differentiation of many cell types. This might explain why the major sources of plasma PAI-1 happen to be differentiated cell types such as adipocytes, skeletal muscles, hepatocytes, vascular endothelial cells and platelets, and therefore warrant further investigation into the role of E2F in mediating PAI-1 gene expression in these cell types.

Elevated levels of PAI-1 in obesity are considered to be at least in part due to direct stimulation of adipocytes by growth factors, cytokines and hormones (e.g., insulin, TNF-α and -β) that are by themselves elevated under conditions of obesity [Dellas and Loskutoff, 2005]. This may be achieved by the enhanced sensitivity acquired by adipocytes to respond to PAI-1 inducing agents. By using peptides that release endogenous E2F (bound to pRB), and thereby restoring the E2F-mediated transcriptional repression, we could desensitise the adipocytes to PAI-1-inducing agents without causing unwanted effects such as cell-cycle re-entry. Although many signals, such as PKB-FOXO, and Erk-AP-1 are known to mediate insulin-induced PAI-1 gene expression, this study shows that E2F is a critical downstream

mediator that plays a decisive role in regulating PAI-1 gene expression.

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