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Serum-free differentiation of 3T3-L1 preadipocytes is characterized by only transient expression of peroxisome proliferator-activated receptor-γ

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Abstract

The adipogenic transcription factor peroxisome proliferator-activated receptor-γ (PPARγ) plays a pivotal role in the regulation of whole body insulin sensitivity. Little, however, is known about hormonal and molecular modulation of PPARγ gene expression. Therefore, we investigated the temporal and conditional expression of PPARγ in a serum-free model of 3T3-L1 adipocyte differentiation. We show here that expression of PPARγ depends on the full set of known adipogenic stimuli and not on a single hormone/agent. Unexpectedly, an indeed marked but only transient peak of PPARγ expression (39 ± 5-fold increase over basal on day 3 after hormonal stimulation) occurs during serum-free adipose conversion. To our knowledge, this finding is novel and probably remained hidden until now because of the common use of serum-containing preadipocyte culture systems. We conclude that maintenance, but not induction, of PPARγ gene expression in vitro must be achieved by one or more still unknown serum component(s).

Keywords: Peroxisome proliferator-activated receptor-γ; 3T3-L1; Adipocyte differentiation; Adipose conversion; Platelet-derived growth factor; Insulin sensitivity; Insulin resistance

Peroxisome proliferator-activated receptor-γ (PPARγ), a well-known key transcription factor in adipocyte differentiation, was recently recognized as a central player in the modulation of whole body insulin sensitivity, and thiazolidinediones (TZD), a class of high affinity ligands for PPARγ promoting PPARγ activation and induction, are clinically used in the treatment of insulin resistance and type 2 diabetes (for review, see [1]). The site of TZD action, however, is still a matter of debate with adipose tissue being one of the most important because of its high level of PPARγ expression. Adipose conversion represents a transcriptionally regulated differentiation program. Among the so far identified transcriptional regulators, the CCAAT/enhancer-binding protein (C/EBP) isoforms α, β, and δ, as well as the nuclear hormone receptor PPARγ are of pivotal importance. According to the actual model (for review, see [2]), C/EBPβ and δ, are the first transcription factors expressed early after hormonal stimulation of adipogenesis. These factors are thought to transactivate the PPARγ gene by binding to its promoter. Newly formed PPARγ is, after heterodimerization with retinoid X receptor and binding of an as yet unknown endogenous lipid ligand, able to induce its own gene, the C/EBPα gene, and, partly in concert with C/EBPα, all genes constituting the adipocyte phenotype. Recently, it was furthermore demonstrated that C/EBPα reinforces PPARγ expression opening the possibility that both transcription factors maintain their expression by positively regulating each other's gene [3]. Whereas this transcriptional cascade is well established now, very little is known about hormonal factors and molecular mechanisms triggering induction and maintenance of PPARγ expression. To investigate this in vitro under defined conditions, i.e., in the absence of unknown and possibly interfering serum factors, we used here the

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formerly described culture system for completely serum-free growth and differentiation of 3T3-L1 preadipocytes [4,5]. In this model, a hormonal cocktail consisting of the adipogenic agents insulin, corticosterone, 3-isobutyl-1-methylxanthine (IBMX), and the major serum mitogen platelet-derived growth factor (PDGF) was shown to induce many if not all characteristics of in vitro adipose conversion including post-confluent mitoses, lipid deposition, induction of the lipogenic marker enzyme α-glycerophosphate dehydrogenase (GPDH), and the typical temporal expression profile of C/EBP isoforms [4–6]. We examined in this system the temporal and conditional expression of PPARγ.

Materials and methods

Cell culture. Culture of 3T3-L1 preadipocytes (ATCC, Manassas, VA, USA) was performed as formerly described in detail [5]. Briefly, cells maintained in newborn calf serum-containing stock cultures were inoculated with 10,000 cells/cm^2, allowed to attach overnight, then washed and grown to confluence in serum-free basal medium (Dulbecco's modified Eagle's medium/Ham's nutrient mixture F-12 (3:1), 100 U/ml penicillin, 0.1 mg/ml streptomycin, 1 μM biotin, 17 μM pantothenate, 2 μg/ml transferrin, and 0.2 mM ascorbic acid) supplemented with 1 μM arachidonic acid, 0.5 nM PDGF (BB-homodimer), and 1 μM insulin for 6 days. Confluent cultures were synchronized by withdrawal of mitogenic stimuli for 48 h (basal medium + 1 nM insulin). Subsequently, adipose conversion was induced (= day 0) with 1 nM insulin, 0.5 μM corticosterone, 0.2 mM IBMX, and 2 nM PDGF. Four days later, the cells were shifted to and maintained until the end of the protocol (= day 8) in basal medium supplemented solely with 1 nM insulin. Non-differentiating control cultures were stimulated with 1 μM insulin, 0.5 μM corticosterone, and 0.2 mM IBMX during the four days induction period. During serum-free culture, the cells were refed every second day.

Determination of adipose conversion. To determine the degree of adipocyte differentiation, the activity of the marker enzyme GPDH was measured on day 8 as described formerly [4]. Cytosolic protein was determined by the Lowry method.

Immunoblot analysis. Total cell lysates were prepared according to the instructions of the antibody supplier (Santa Cruz), the protein contents were measured by the Bradford method. For detection of PPARγ, 50 μg samples were electrophoresed through a 8% SDS–polyacrylamide gel, transferred onto nitrocellulose (Schleicher & Schuell, Dassel, Germany) and probed for one hour with 0.5 μg/ml of the monoclonal antibody E-8 (Santa Cruz Biotechnology, Heidelberg, Germany). Immunoreactive protein was visualized by the ECL detection method (Amersham Life Science, Braunschweig, Germany) and quantified by videodensitometry as described earlier [6].

Results and discussion

Time course of PPARγ expression

Adipose conversion of serum-free cultured murine 3T3-L1 preadipocytes depends not only on insulin,

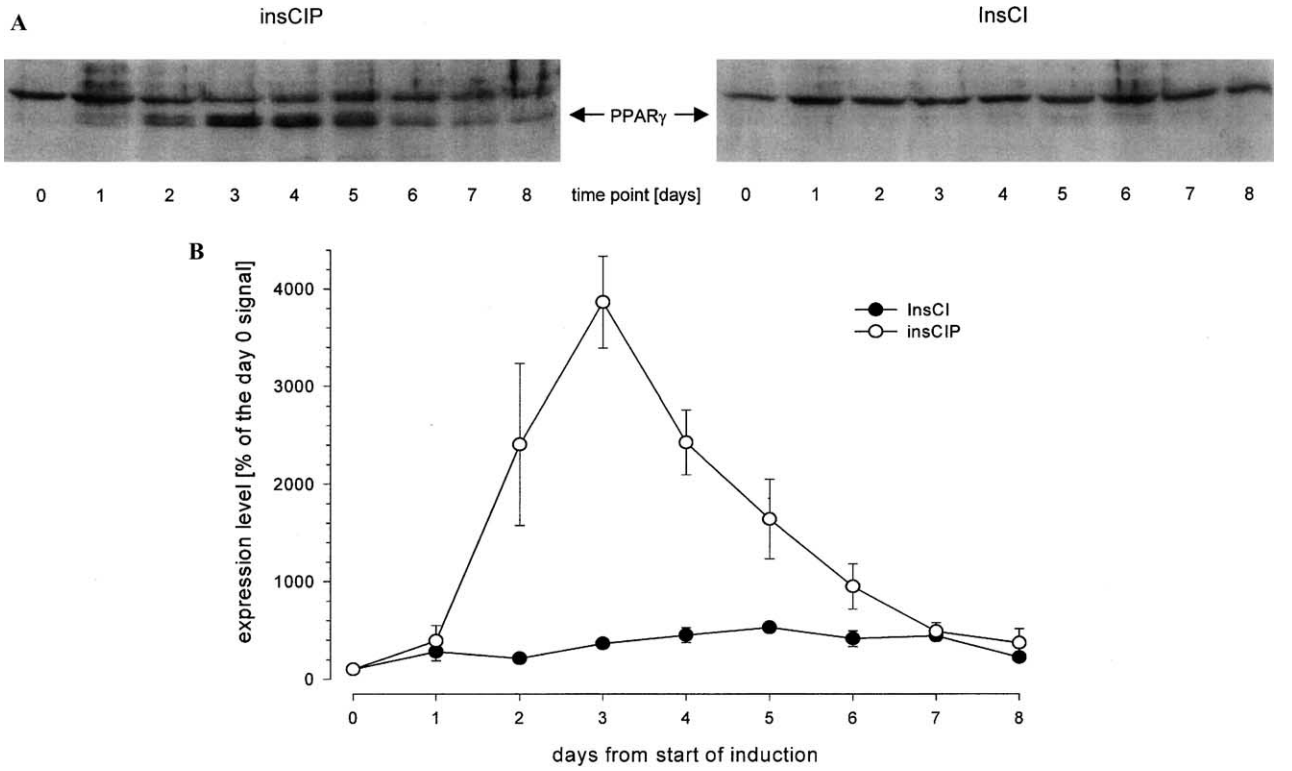

Fig. 1. Time course of PPARγ expression in differentiating and non-differentiating 3T3-L1 preadipocytes. Cells were cultured and stimulated as described in Materials and methods. (A) Representative immunoblots. (B) Quantitative evaluation of specific signals by videodensitometry. The data are presented as means ± SEM of four independent experiments (Ins = 1 μM insulin, ins = 1 nM insulin, $C = 0.5$ μM corticosterone, $I = 0.2$ mM IBMX, $P = 2$ nM PDGF).

glucocorticoids, and cyclic adenosine-monophosphate-elevating agents like IBMX, but also on special growth factors like PDGF or epidermal growth factor [4,5]. Insulin-like growth factor-1 or a high concentration of insulin (1 μM), known to act via the insulin-like growth factor-1 receptor as well [7], were shown to be unable to induce 3T3-L1 differentiation in concert with corticosterone and IBMX [4]. Accordingly, confluently grown 3T3-L1 preadipocytes stimulated with 1 μM insulin, corticosterone, and IBMX show scarce lipid deposition (see [4]), low GPDH activity (means ± SEM: 5.5 ± 1.5 mU/well and 384.2 ± 40.9 mU/mg on day 8 after start of induction; $n = 4$), and only marginally elevated levels of PPARγ (Figs. 1 and 2). Replacement of high insulin (1 μM) by PDGF and low insulin (1 nM) within the induction cocktail, however, induces adipogenesis as shown earlier [5] and as mirrored by elevated GPDH activities (means ± SEM: 84.0 ± 3.0 mU/well and 1286.7 ± 106.4 mU/mg on day 8 after start of induction; $n = 4$). These conditions provoked a 38.6 ± 4.7-fold increase (mean ± SEM) of PPARγ expression (Fig. 1). Interestingly, the antibody applied for these studies detects a doublet of closely migrating bands differing by less than 1 kDa and representing probably phosphorylated and unphosphorylated forms of PPARγ2, the predominant PPARγ isoform in adipocytes. Furthermore, we suppose that the 3 kDa smaller isoform PPARγ1 is below the detection limit of our method. By means of a blocking peptide, the identity of the doublet with PPARγ was verified, whereas the higher migrating band was unmasked as an unspecifically reacting protein (not shown). PPARγ expression reached a sharp peak on day 3 and, thereafter, declined to basal levels. This finding was unexpected since PPARγ expression was reported earlier to remain sustained until the end of commonly used serum-based differentiation regimens [8–10]. Therefore, the decline of PPARγ expression in serum-free culture might reflect the loss of unknown serum factors necessary for the maintenance of PPARγ expression. The identification and characterization of these physiological serum components should further advance our understanding of regulated PPARγ expression and PPARγ-mediated whole body insulin sensitivity.

Dependence of PPARγ expression on different adipogenic inducers

Induction of PPARγ in serum-free cultured 3T3-L1 preadipocytes depends on the complete set of adipogenic inducers including PDGF (Fig. 2). Unlike high

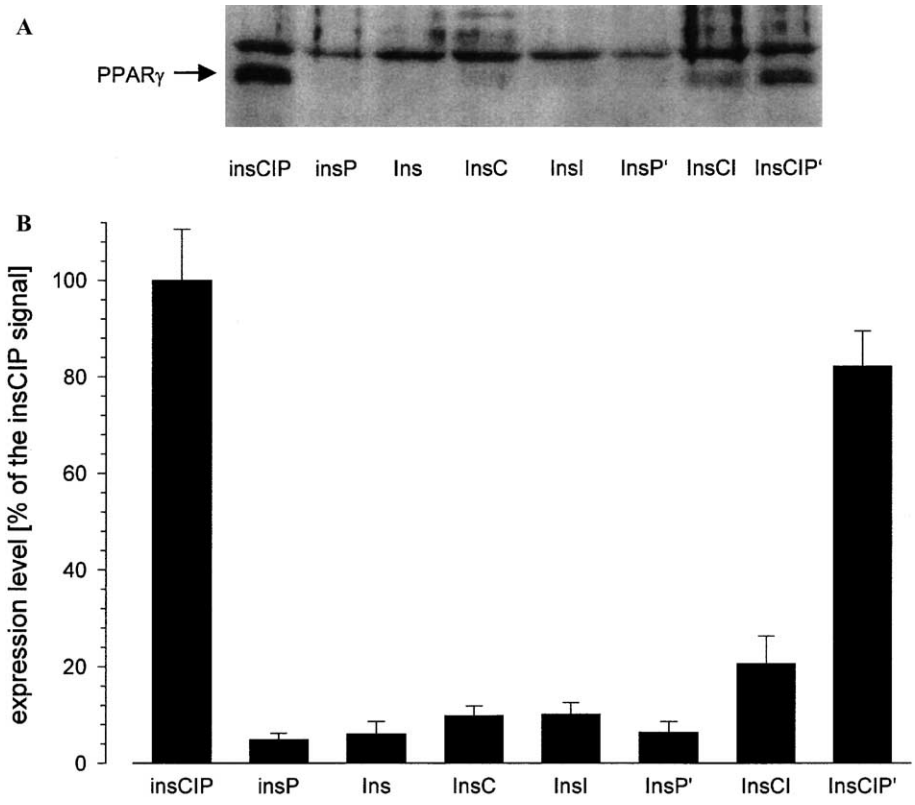

Fig. 2. Dependence of PPARγ expression on adipogenic stimuli. Cells were cultured and stimulated as described in Materials and methods. PPARγ expression was studied in cell lysates prepared on day 3 after start of induction. (A) Representative immunoblot. (B) Densitometric quantitation of specific signals. The data are presented as means ± SEM of four independent experiments (Ins = 1 μM insulin, ins = 1 nM insulin, $C = 0.5$ μM corticosterone, $I = 0.2$ mM IBMX, $P = 2$ nM PDGF). In the presence of high insulin, only 1 nM PDGF ($= P'$) was used to avoid a too strong mitogenic response known to inhibit adipose conversion.

concentrations of insulin (1 μM), PDGF is able to induce maximal levels of PPARγ in concert with corticosterone and IBMX. Since both factors act as potent survival factors for 3T3-L1 cells, this finding provides further evidence that PDGF has additional adipogenesis-promoting functions as observed earlier [5]. In this respect, it is of importance that PDGF was shown to be a strong inducer of the C/EBPβ isoform LAP in serum-free cultured 3T3-L1 cells [6]. In the light of the favourable effects of PPARγ activation and induction on insulin sensitivity, it would be important to intensify studies on hormones stimulating PPARγ induction and to further extend these studies to primary human adipocytes as well as to other insulin-responsive human cell types.

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