Evidence for a novel regulatory pathway activated by (carba)prostacyclin in preadipose and adipose cells

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Abstract Prostacyclin, one of the major prostanoids generated in adipose tissue, has been previously described as an autocrine/paracrine adipogenic effector, acting, in preadipose cells, by means of cAMP and free Ca^{2+} as cell surface receptor-mediated messengers. The present study presents evidence for the first time that its stable analogue, carbaprostacyclin, is unique among prostanoids in regulating the expression of two differentiation-dependent genes in preadipose and adipose cells in a way distinct from that elicited by its cell surface receptor. This regulation is likely mediated by some member(s) of the peroxisome proliferator-activated receptor family and suggests that prostacyclin behaves as an intracrine effector of adipose cell differentiation.

Key words: Prostanoid; Prostacyclin; Angiotensinogen; Fatty acid binding protein; Peroxisome proliferator activated receptor; Adipose tissue

1. Introduction

The terminal differentiation of preadipose into adipose cells is triggered by a few adipogenic hormones [1]. Among prostanoids, a potent and specific adipogenic role has been delineated for carbaprostacyclin ($cPGI_2$) and other stable analogues of prostacyclin (PGI_2) [2–4], which is one of the major metabolites of arachidonic acid in both preadipose and adipose cells [5–8]. On a short-term basis both PGI_2 and its stable analogue $cPGI_2$ play a similar dual role in eliciting elevation of intracellular cAMP and free Ca^{2+} [9,10] and, in addition, are equally active on a long-term basis in triggering terminal differentiation of preadipose to adipose cells ([2] and J. Aubert et al., unpublished results). $cPGI_2$ has indeed been shown to specifically promote terminal differentiation not only of preadipocytes from clonal lines but also that of adipose precursor cells isolated from murine or human adipose tissue and maintained in primary culture under chemically defined, serum-free conditions [4,11]. Moreover, PGI_2 behaves as an autocrine/paracrine effector of adipose cell differentiation in vitro by means of binding to its cell surface receptor [3,4,12,13]. Recent studies, using adipose tissue explants or in situ microdialysis of fat pads, have shown that this prostaglandin (PG), known to be present in the interstitial fluid of adipose tissue [14], is able to recruit adipose precursor cells and to trigger the formation of new fat cells (C. Darimont et al., unpublished results).

After binding to their cell surface receptors, the ability of PGI_2 and $cPGI_2$ to activate adenylate cyclase via a Gs-dependent mechanism in preadipose but not in adipose cells [15], coupled to their ability to increase intracellular free Ca^{2+} [4,14], was thought to account for their potent and specific adipogenic property. Quite recently, 15-deoxy-$\Delta^{12,14}$ PGJ_2, a PGJ_2 metabolite, has been shown to bind to peroxisome proliferator-activated receptor γ (PPARγ), a member of the nuclear receptor superfamily of ligand-activated transcription factors, and to promote adipose cell differentiation [16–18]. So far, no evidence of synthesis of 15-deoxy-$\Delta^{12,14}$ PGJ_2 has been given in either preadipose or adipose cells, raising concern about the physiological significance of this finding. Furthermore, PPARα and PPARδ, also reported to be present in preadipose and adipose cells, but more ubiquitously expressed than PPARγ [19], have also been shown to be activated by various prostanoids [17,18,20–22]. In those studies, the activation potency of a given effector was depending on the nature of the recipient cell and on that of the combination of expression vector and reporter gene. To add further complications $cPGI_2$, reported to be an activator of all three PPAR isoforms in transactivation assays, exhibited an adipogenic activity in NIH fibroblasts stably transfected with PPARα and PPARγ but not with PPARδ [22]. Altogether, these results strongly suggest that activators modulate the availability and/or the formation of intracellular ligands. As both events may differ in a particular cellular context, studies using model target cells, i.e. preadipose and adipose cells from clonal lines, are indeed required. The validity of using such cells has been previously shown, since they behave as authentic precursor cells of adipocytes both in vitro and in vivo [23,24], similarly to precursor cells isolated from adipose tissue [25].

These various observations have prompted us to investigate, in Ob1771 and 3T3-F442A preadipose and adipose cells, the regulation by $cPGI_2$ of the expression of two genes already shown to respond to long-chain natural or non-metabolized fatty acids as well as peroxisome proliferators, i.e. genes encoding for angiotensinogen (AT) (I. Safonova et al., submitted for publication) and adipocyte fatty acid binding protein (a-FABP) [26,27], the promoter region of which was known to contain a peroxisome proliferator response element [28]. The results reported herein show that $cPGI_2$ is unique among PGs to activate the expression of AT and a-FABP genes in these cells and that its effect is distinct from that taking place via its cell surface receptor.

2. Materials and methods

2.1. Cell culture

Cells of Ob1771 [29] and 3T3-F442A [30] clonal lines were plated at a density of 2×10^3 cells per cm^2 and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 8% fetal bovine serum, 200 units/ml of penicillin, 50 μg/ml of streptomycin, 33 μM biotin, and 17 μM pantothenate (referred to as standard medium). Under

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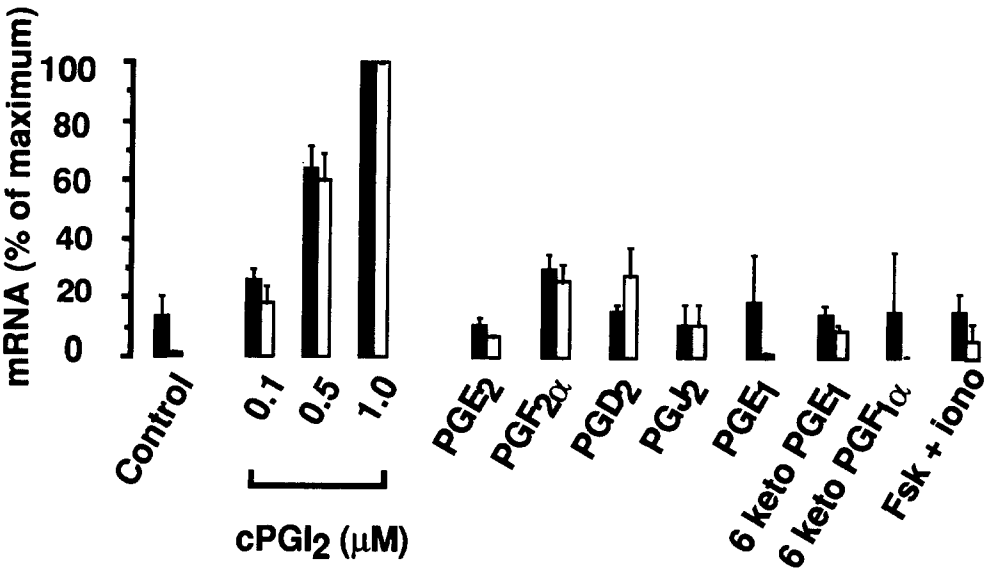

Fig. 1. Accumulation of AT and a-FABP mRNAs in Ob1771 preadipose cells exposed to various prostaglandins or to cAMP and Ca^{2+} elevating drugs. One-day post-confluent preadipose cells cultured as described in Section 2 were maintained for 24 h in IT medium alone (control) or supplemented either with increasing concentrations of $cPGI_2$ as indicated, or with various prostaglandins (all present at a concentration of 1 μM) or with a mixture of 10 μM forskolin and 0.1 μM ionomycin (Fsk+iono). RNAs were then extracted and analyzed (20 μg/lane) as described in Section 2. The intensities of the signals were normalized to those of β-actin and expressed by taking as 100% the maximal response obtained for each probe. The data are the means ± S.E.M. of values obtained in three separate experiments. Black columns correspond to AT and white columns to a-FABP mRNA.

these conditions, confluence (day 0) was reached within 5 days. Unless otherwise stated 1 day post-confluent cells, i.e. lipid-free preadipose cells known to express early markers of differentiation only [1], were used. Triacylglycerol-containing differentiated cells, able to express late markers of differentiation as well [1], were also used in some experiments. These latter were obtained by feeding confluent Ob1771 as well as 3T3-F442A cells chronically with standard medium described above containing 17 nM bovine insulin plus 2 nM triiodothyronine, and supplemented with 10 nM dexamethasone and 100 μM 3-isobutyl-1-methylxanthine during the first 3 days. Media were changed every other day.

For experimental purposes, both undifferentiated preadipose cells and differentiated adipose cells were transferred to a serum-free medium consisting of DMEM supplemented with 850 nM bovine insulin and 10 μg/ml human transferrin. This serum-free, chemically defined medium (referred to as IT medium) was supplemented or not with the various effectors and for periods of time indicated in the legend of figures.

2.2. RNA analysis

RNAs were prepared by the guanidium thiocyanate technique [31]. For Northern blot analysis, 20 μg total RNAs were separated by electrophoresis on 1.2% (w/v) agarose gel containing 1.1 M formaldehyde and transferred onto a Hybond-N^+ membrane (Amersham, France). Hybridizations were performed for 24 h at 65°C in 0.5 M phosphate buffer, pH 7.2, containing 5% SDS and 10^6 cpm/ml of randomly primed ^{32}P-labeled cDNA probes. The autoradiographs were quantitated using a Fujix phosphorimager. All results were normalized to β-actin signals.

2.3. Angiotensinogen protein assay

Ob1771 and 3T3-F442A cells, grown and previously induced to differentiate as described above (2 cm^2 culture wells), were thoroughly washed three times (twice rapidly and once for 1 h) with IT medium. They were then incubated at 37°C for 24 h in 1 ml of fresh IT medium containing or not an additional effector. The culture medium was collected in the presence of 1% bovine serum albumin and a cocktail of anti-proteolytic agents including 1 mM paramethylsulfonylfluoride, 5 mM EDTA, 1 mM hydroxyquinoline and 10 μM captopril. The amount of AT present in suitable aliquots of culture medium was determined, after its total conversion to angiotensin I (AngI) in the presence of an excess of mouse renin, by measuring AngI with a radioimmunoassay [32]. Renin from mouse submaxillary gland was prepared according to the method of Jacobsen and Poulsen [33].

2.4. Materials

Culture media were obtained from Gibco (Cergy-Pontoise, France) and fetal bovine serum from Seromed (Berlin, Germany). All other cell culture products were obtained as previously reported [9,10]. [α-^{32}P]dCTP, random priming kit, Hybond membranes and products used for the radioimmunoassay of AT were purchased from Amersham (Les Ulis, France). Restriction enzymes were from Eurogentec (Seraing, Belgium). Actinomycin D, cycloheximide, dexamethasone, forskolin and ionomycin were purchased from Sigma Chimie (Saint Quentin Fallavier, France). All prostanoids were products from Cayman Chemicals (SpiBio, Massy, France). RU 38486 was a kind gift from Roussel Uclaf. The sources of the various cDNAs used in this study are acknowledged in the corresponding section.

3. Results

As shown in Fig. 1, Ob1771 preadipose cells, maintained in IT medium, were able to respond specifically, rapidly (within 24 h) and dose-dependently to $cPGI_2$ in accumulating high levels of AT and a-FABP mRNAs. Apart from $PGF_{2\alpha}$ and PGD_2, which slightly enhanced mainly the a-FABP signal, other PGs such as PGE_2, PGJ_2, PGE_1, 6-keto-PGE_1 and 6-keto-$PGF_{1\alpha}$, when present at 1 μM, were unable to exert a significant effect on the level of both AT and a-FABP mRNA. The metabolites of PGJ_2, Δ^{12}-PGJ_2 and 15-deoxy-$\Delta^{12,14}$-PGJ_2, were also found to be inactive (not shown). It is striking that PGE_1 and 6-keto-PGE_1 were inactive despite their binding to the same cell surface receptor as PGI_2 [34,35], and their ability to alter intracellular cAMP and free calcium levels [10] and to induce the terminal differentiation of Ob1771 preadipose cells, although to a lower extent than $cPGI_2$ [2]. Moreover, simultaneous exposure to an adenylate cyclase agonist (forskolin) and a calcium ionophore (ionomycin), which are able to mim-

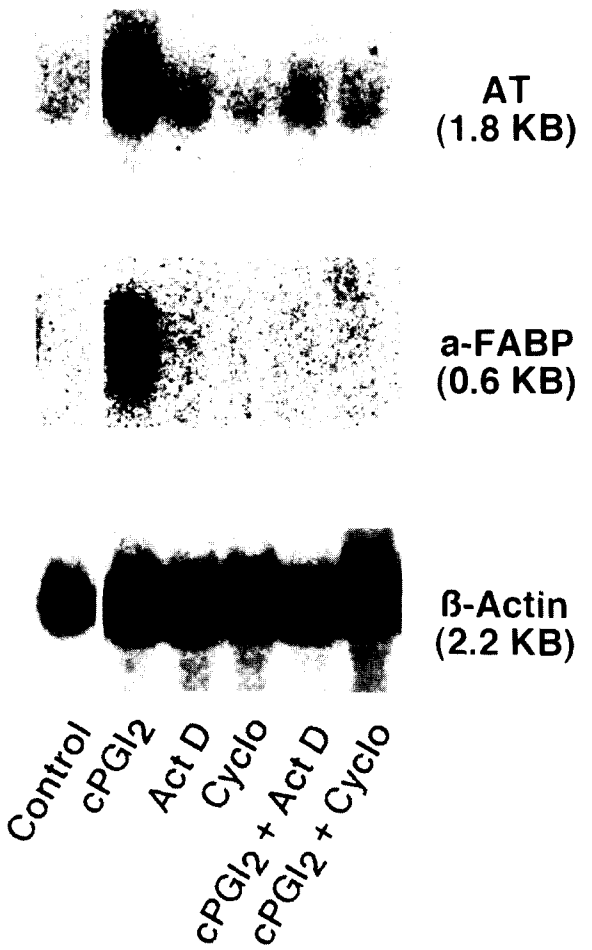

Fig. 2. Effect of actinomycin D and cycloheximide on AT and a-FABP mRNA levels in Ob1771 preadipose cells exposed to $cPGI_2$. One-day post-confluent preadipose cells were maintained for 20 h in IT medium alone (control) or supplemented with 1 μM $cPGI_2$, 2 μg/ml actinomycin D (Act D), 1 μg/ml cycloheximide (Cyclo) or a combination of $cPGI_2$ and these latter compounds ($cPGI_2$+Act D and $cPGI_2$+Cyclo, respectively). 15 h later, RNAs were extracted and analyzed (20 μg/lane) as described in Section 2. The results are representative of three separate experiments.

ic the intracellular signals triggered by $cPGI_2$ after binding to its cell surface receptor [4] and also to lead to terminal differentiation of preadipose to adipose cells, did not change AT or a-FABP mRNA levels. Therefore, the specific stimulating effect of $cPGI_2$ on AT and a-FABP genes appeared to be mediated by a mechanism distinct from the cell surface receptor signalling pathway.

As shown in Fig. 2, the accumulation of AT and a-FABP mRNAs in preadipose cells exposed to 1 μM $cPGI_2$ for 15 h was abolished in the presence of actinomycin D or cycloheximide, suggesting that $cPGI_2$ regulates the expression of these genes by means of transcriptional activation and requires de novo protein synthesis.

Besides arachidonic acid, PGI_2 and PGI_2 analogues, glucocorticoids are the only hormones able to trigger terminal differentiation of Ob1771 preadipose cells under serum-free culture conditions [1,3,12]. These hormones are also able to activate transcription of several genes expressed during the differentiation process, including AT (J. Aubert et al., unpublished experiments) and a-FABP [26]. A possible interaction of $cPGI_2$ with the glucocorticoid nuclear receptor, if any, had to be excluded. Northern blots presented in Fig. 3 show clearly that the glucocorticoid antagonist RU 38486 completely abolished the increase in AT and a-FABP mRNAs induced by a maximally effective concentration of dexamethasone, whereas it had no effect on that induced by $cPGI_2$. These results demonstrate that AT and a-FABP gene expression were independently regulated by glucocorticoids and $cPGI_2$. Furthermore, in the light of the small increase in a-FABP mRNA elicited by 0.1 μM dexamethasone, these results would indicate a preferential regulation of a-FABP gene expression by $cPGI_2$ as compared to glucocorticoids.

The ability of PGI_2 and $cPGI_2$ to increase intracellular cAMP has been shown to affect cells at the preadipose state exclusively [15], strongly suggesting that cell surface receptors and/or the coupling mechanism that mediate this biological response disappeared during the differentiation process. Thus the regulation of AT and a-FABP gene expression as well as AT secretion, if any, was studied in differentiated Ob1771 and 3T3-F442A adipose cells (Fig. 4). For that purpose, prior decrease of both mRNAs was obtained by a 20 h preincubation period in IT medium. The results of Fig. 4A show that differentiated cells responded to $cPGI_2$ by an increase in AT and a-FABP mRNA levels in a dose-dependent

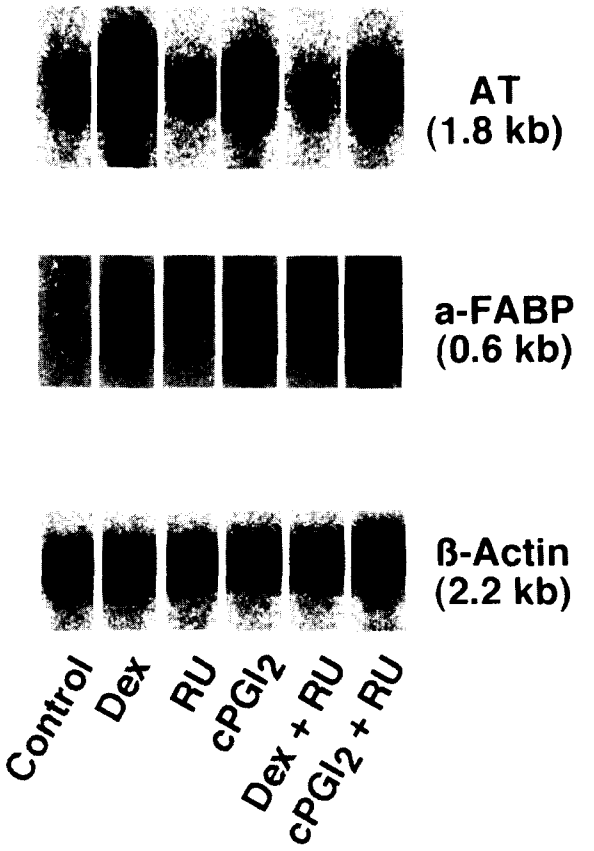

Fig. 3. Differential modulation of AT and a-FABP mRNA accumulation by dexamethasone and $cPGI_2$ in Ob1771 preadipose cells. One-day post-confluent preadipose cells were maintained for 24 h in IT medium alone (control) or supplemented with 0.1 μM dexamethasone (Dex), 1 μM $cPGI_2$, 0.1 μM RU 38486 (RU) or a combination of this latter compound with dexamethasone or $cPGI_2$ (Dex+RU and $cPGI_2$+RU). RNAs were then extracted and analyzed (20 μg/lane) as described in Section 2. The results are representative of three separate experiments.

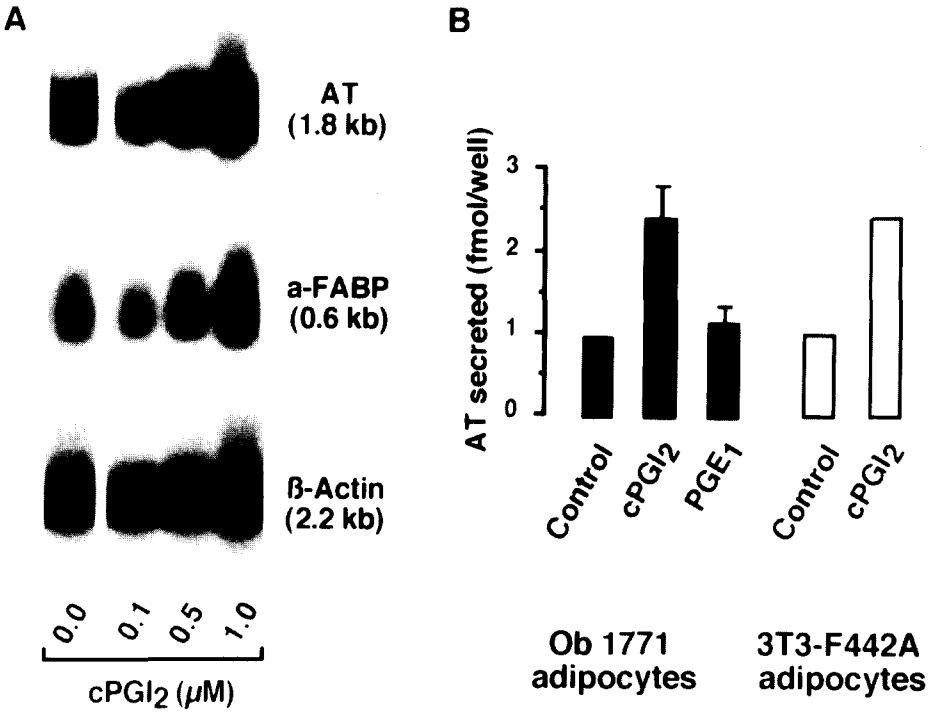

Fig. 4. Induction of AT and a-FABP mRNA and AT secretion in Ob1771 and 3T3-F442A adipose cells by $cPGI_2$. Ob1771 or 3T3-F442A cells, grown and maintained under the culture conditions described to induce their differentiation (see Section 2) were transferred to IT medium for 20 h. They were then fed with fresh IT medium that was supplemented with increasing concentrations of $cPGI_2$ as indicated for Ob1771 cells in A or with either 1 μM $cPGI_2$ or PGE_1 for Ob1771 and 3T3-442A adipocytes in B. 24 h later, RNAs were extracted and analyzed (20 μg/lane). The result of this analysis representative of three separate experiments is shown in A. The amounts of AT, present in the culture medium of adipocytes exposed during 24 h to IT medium alone or supplemented with either 1 μM $cPGI_2$ or PGE_1, measured by radioimmunoassay (see Section 2) are shown in B. The data are the means ± S.E.M. of values obtained in three separate experiments.

manner. Furthermore, in agreement with the fact that AT mRNA expression is accompanied by AT synthesis and secretion (J. Aubert et al., unpublished results), 1 μM $cPGI_2$ was also able to give rise to a 2.5-fold increase in the amount of AT present in the culture medium of both Ob1771 and 3T3-F442A differentiated cells (Fig. 4B). Once again, PGE_1 failed to mimic $cPGI_2$ in enhancing AT secretion into the culture medium.

4. Discussion

Both PGI_2 and the stable prostanoid $cPGI_2$, like glucocorticoids, are known to control on a long-term basis the terminal differentiation of growth-arrested Ob1771 preadipose cells ([2] and J. Aubert et al., unpublished results). The regulation of AT and a-FABP gene expression by $cPGI_2$ on a medium-term basis appears distinct from the differentiation process per se as it occurs many days earlier than terminal differentiation and under culture conditions which are insufficient to induce terminal differentiation [1,3,12]. This regulation is rather specific as $cPGI_2$ appears to be the sole active PG and it is not confined to Ob1771 cells since it occurs also in 3T3-F442A cells. It is unlikely that this specific effect might be due to the stability of $cPGI_2$ as compared to that of other PGs. First, stable PGE_1, PGE_2, $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and 6-keto-PGE_1 are inefficient in inducing AT and a-FABP gene expression (Fig. 1). Second PGI_2, which shares with PGD_2 and PGJ_2 a poor chemical stability, is the only prostanoid able to trigger the terminal differentiation of preadipose to adipose cells during which AT and a-FABP genes are induced ([2] and J. Aubert et al., unpublished results).

The salient feature of this regulation by $cPGI_2$ is its clear dissociation from events controlled by the 'classical' cell surface receptor of PGI_2 (IP receptor) [36,37]. Three arguments are in favor of this conclusion: (i) the inability of two PGs to mimic the $cPGI_2$-induced AT and a-FABP gene expression, namely PGE_1 and 6-keto-PGE_1, despite the fact that both are ligands of the IP receptor [34,35], alter intracellular cAMP and free Ca^{2+} levels [4,10] and exhibit weak adipogenic properties [2], (ii) the inability of forskolin and ionomycin to control AT and a-FABP gene expression despite their ability to induce terminal differentiation [4] and (iii) the persistence of the regulation of AT and a-FABP gene expression by $cPGI_2$ in differentiated cells, once they become unable to generate cAMP upon exposure to this prostanoid [15]. In this latter case, a possible response due to undifferentiated cells still remaining in the population can be ruled out since AT secretion is very weak, if any, in preadipose cells (I. Safonova et al., submitted for publication).

Among hypotheses to explain the gene effect of $cPGI_2$, the implication of other plasma membrane prostanoid receptors is rather unlikely. Both FP and DP receptor can be excluded since their most specific ligands, $PGF_{2\alpha}$ and PGD_2 respectively, fail to activate AT and a-FABP gene expression. One could argue that the EP1 receptor isoform is implicated as it is also able to bind iloprost, a stable analogue of PGI_2 [36]. This hypothesis can also be excluded as its most specific ligands (PGs of the E series, i.e. PGE_1 and PGE_2) are inactive at the

gene level. This conclusion can be extended for the same reason to other characterized EP receptor isoforms (EP2 and EP3 receptors).

Although the implication of plasma membrane receptors different from known prostanoid receptors cannot be ruled out, it is proposed that the effect of $cPGI_2$ at the gene level is mediated by members of the PPAR family. This proposal is supported by several observations: (i) AT and a-FABP gene expression is regulated in preadipose cells by non-metabolizable and natural long-chain fatty acids, through activation of PPAR δ and/or PPAR γ which are respectively present at high and low levels in Ob1771 preadipose cells and which both accumulate in differentiated adipose cells ([26,27] and I. Safonova et al., submitted for publication), (ii) PGI_2 analogues, especially $cPGI_2$, have been reported in transactivation assays to activate not only PPARα [21] but all PPAR isoforms in the range of concentrations required in the present study (0.025-10 μM) [21] and (iii) PPAR activators, including prostanoids and $cPGI_2$, are able to induce adipogenesis in transfected fibroblasts overexpressing PPARα or PPARγ within the same range of concentrations (0.5–10 μM) [17,18,20,22].

It should be recalled that, besides PGE_2 and trace levels of $PGF_{2\alpha}$, PGI_2 is the only arachidonic acid metabolite whose production has been clearly demonstrated to occur in preadipose and adipose cells both in vitro [5–8] and in vivo [14]. Since neither PGE_2 nor $PGF_{2\alpha}$ was shown to play a direct role at the gene level as well as in adipogenesis [2,4,10,15], it appears that PGI_2 is the most likely prostanoid candidate to regulate adipose cell gene expression and differentiation.

In summary, the novel regulatory pathway described herein for $cPGI_2$ may be relevant to an intracrine effect of PGI_2 generated from arachidonic acid in preadipose cells [5,6]. This gene effect might implicate PPARs. If this were so, like some members of the steroid hormone receptor family reported to be phosphoproteins [38], a control of the phosphorylation state and activity of PPARs in response to prostacyclin could be postulated in preadipose cells.

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