Stimulation of adipose differentiation related protein (ADRP) expression by ibuprofen and indomethacin in adipocyte precursors and in adipocytes

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Adipose differentiation related protein (ADRP) is a 50 kDa protein expressed at high level in differentiated adipocytes. ADRP expression is very low in undifferentiated adipocytes and increases rapidly and dramatically as the cells undergo adipose differentiation. In the present study, we demonstrate that ADRP expression at the mRNA and protein level is stimulated in adipocyte precursor cells in a time- and dose-dependent fashion by treatment with cyclooxygenase inhibitors, particularly indomethacin and ibuprofen. Lipoxigenase inhibitors such as AA861 and nordihydroguaiaretic acid were ineffective. Stimulation of ADRP expression was observed with 10^{-5} M ibuprofen but

maximal stimulation required a concentration of 3×10^{-4} M. Nuclear run-on experiments indicated that indomethacin or ibuprofen stimulated the transcription of the ADRP gene in undifferentiated adipocytes. In addition to stimulating the induction of ADRP in undifferentiated cells, ibuprofen and indomethacin also stimulated the level of ADRP mRNA and protein in differentiated adipocytes. These experiments provide new information on the regulation of ADRP, an early inducible gene in the adipocyte differentiation programme in adipocyte precursors and in adipocytes and identify a new target for cyclooxygenase inhibitor action during adipocyte differentiation.

INTRODUCTION

Adipocyte differentiation is characterized by a co-ordinate increase in gene expression. In most cases, this increase can be accounted for by activation of gene transcription [1]. Studies of adipose differentiation have been facilitated by the availability of established adipogenic cell lines that can be induced to differentiate upon treatment with mixtures of hormonal stimulants. In particular, these cell lines have provided useful systems for the identification of novel gene products induced during the onset of differentiation. Adipose differentiation related protein (ADRP) is a 50 kDa protein encoded by a gene originally cloned from a differentiated 1246 adipocyte cDNA library by differential hybridization [2]. It was shown that ADRP mRNA was 1.7 Kb in size and was induced 50- to 100-fold a few hours after the initiation of adipose differentiation in 1246 cells, making it an early marker of the process [3]. Similarly, the 50 kDa ADRP protein was undetectable in undifferentiated cells and its expression increased as the cells differentiated. Tissue distribution of ADRP mRNA expression in mice indicated that ADRP mRNA was expressed at high level in adipose tissue [3]. In addition, recent experiments have shown that ADRP is also found in many different types of cells and tissues that accumulate or synthesize lipids, although at a lower level than in adipose tissue [4]. Moreover, ADRP expression has been found in the liver of mice treated with the carnitine palmitoyltransferase I inhibitor, etomoxir, which caused neutral lipid accumulation in the organ [5], and as a component of milk globules [6]. These studies indicate the possible role of ADRP in the formation or stabilization of lipid droplets in adipocytes and other cells. Thus it is important to identify the factors that regulate the expression

of the ADRP. Since ADRP expression is induced during the onset of the differentiation programme, it is of interest to investigate this in adipocyte precursors.

The effect of prostaglandins (PG) on adipocyte differentiation has been widely studied in adipogenic cell lines and in adipocyte precursors in primary culture and has been shown to be multiple and complex. Reports in the literature suggest that several different prostaglandins have a marked effect on adipose differentiation. For example, it has been shown that carboxyprostaglandin (PGI₂) stimulates the differentiation of Ob1771 cells [7] and 15-deoxy- $\Delta^{12,14}$ PGJ₂ was shown to promote the efficient conversion of mesenchymal cells to adipocytes by binding to the peroxisome-proliferator-activated receptor γ (PPAR γ), a key regulator of adipose differentiation [8–10]. In contrast, PGF₂ α and 9 α ,11 β -PGF₂ α have been shown to inhibit adipose differentiation of adipogenic cell lines and adipocyte precursors in primary culture [11–13]. Studies with primary cultures of rat adipocyte precursors have also shown that PGF₂ α stimulated the expression transforming growth factor- α [14], which also inhibits adipose differentiation, and increased epidermal growth factor receptor internalization [15], thereby providing an amplification mechanism by which differentiation is negatively modulated in physiological conditions during adipose tissue development.

The effect of cyclooxygenase inhibitors on adipose differentiation has also been investigated. It was found that indomethacin stimulated the differentiation of a variety of cell lines including TA1 [16], 1246 [11], 3T3-L1 [17,18] and C3H10T1/2 [19]. However, the fact that the concentrations of indomethacin required to stimulate differentiation were two orders of magnitude higher than those required to inhibit PG production

Abbreviations used: ADRP, adipose differentiation related protein; FAB, fatty acid binding protein; LPL, lipoprotein lipase; NDGA, nordihydroguaiaretic acid; PG, prostaglandins; PPAR γ , peroxisome proliferator activated receptor γ ; RPL32, ribosomal protein L32.

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suggested that the mechanism by which indomethacin stimulated differentiation was distinct from its cyclooxygenase inhibitory effect [16]. Recently, it was found that indomethacin and other cyclooxygenase inhibitors were ligands and activators of PPAR γ [19], a key regulator of adipose differentiation [20–22], thereby providing a novel mechanism for the adipogenic action of cyclooxygenase inhibitors.

We have investigated the hormonal control of ADRP expression and, in the present paper, we show that cyclooxygenase inhibitors are potent stimulators of ADRP mRNA and protein expression both in adipocyte precursors and in adipocytes.

EXPERIMENTAL

Materials

Dulbecco's modified Eagle's medium, Ham's F12 and fetal bovine serum were purchased from Life Technologies (Grand Isle, NY, U.S.A.). Bovine pancreatic insulin, human plasma transferrin, bovine fetuin, indomethacin, ibuprofen and sodium bicarbonate were from Sigma (St. Louis, MO, U.S.A.). Naproxen and nordihydroguaiaretic acid (NDGA) were from Cayman (Ann Harbor, MI, U.S.A.). RNazol was from CinnaBiotech (Friendswood, TX, U.S.A.). AA861 was kindly given by Dr. Hidekazu Sawada (Takeda Pharmaceutical Company, Osaka, Japan). Tissue culture plasticware was from Becton Dickinson (Franklin Lakes, NJ, U.S.A.).

Cell culture and adipose differentiation

The mouse 1246 adipogenic cell line was used in all experiments. 1246 stock cells were maintained in DME/F12 nutrient medium (Dulbecco's modified Eagle's medium/Ham's F12 medium, 1:1) supplemented with 10% (v/v) fetal bovine serum. The differentiation of 1246 cells in serum-free defined medium was carried out as described previously [2]. For assaying the effect of inhibitors of arachidonate metabolism on adipocyte precursors, 1246 cells were cultivated in defined medium, consisting of DME/F12 medium supplemented with bovine pancreatic insulin (10 μ g/ml), human plasma transferrin (10 μ g/ml) and bovine fetuin (250 μ g/ml) until day 4, when they reached confluency. At that time, the medium was changed to fresh, defined medium and the inhibitors indomethacin and ibuprofen (each 3×10^{-5} M), naproxen (3×10^{-4} M), AA861 and NDGA (3×10^{-6} M) were added. All inhibitors were freshly prepared as 1000-fold concentrated solutions in ethanol. Control cells were treated with an equivalent volume of ethanol only.

RNA isolation and Northern-blot analysis

Total RNA was extracted by the RNazol method from triplicate 60-mm plates of 1246 cells cultivated in defined medium. mRNA expression for ADRP was examined by Northern-blot analysis with 10–15 μ g of total RNA as described previously [2]. mRNA expression for ribosomal protein L32 (RPL32) was used as an internal standard for equal RNA loading. cDNAs for ADRP and RPL32 were labelled by the random priming reaction. Hybridization and washing conditions have been described previously [2]. Dried filters were exposed to X-ray films at -70°C with intensifying screens. Signals obtained by autoradiography were quantified by densitometric analysis (Bio-image, Millipore, Bedford, MA, U.S.A.) and normalized to the levels of RPL32 used. The experiments were repeated at least three times. Values were expressed as fold induction above control untreated cells $\pm$ S.E.M.

Nuclear run-on experiments

Nuclear run-on assays were performed according to a modification [23] of the method described by Ausubel et al. [24]. Briefly, 4 to 7×10^7 confluent, undifferentiated 1246 cells treated with or without cyclooxygenase inhibitors were washed three times with ice-cold PBS and detached with a cell scraper. The cells were collected by centrifugation at 200 g for 5 min, resuspended in 8 ml of lysis buffer [10 mM Tris/HCl (pH 7.4)/10 mM NaCl/3 mM MgCl_2 /0.5% (v/v) Nonidet P-40], incubated on ice for 5 min and centrifuged at 500 g for 5 min. After three washes in lysis buffer, the nuclei were resuspended in 160 μ l of storage buffer [50 mM Tris/HCl (pH 8.3)/40% (v/v) glycerol/5 mM MgCl_2 /0.1 mM EDTA] and were either used immediately or stored at -70°C until used. The transcription assay was initiated by adding 20 μ l of $10 \times$ run-on stock solution (6 mM ATP/3 mM GTP/3 mM CTP/30 mM dithiothreitol/12 mM MgCl_2 /800 mM KCl) and 200 μ Ci of [α - ^{32}P]UTP (3000 Ci/mmol) to the nuclei suspension (160 μ l). After 30 min incubation at 30°C , nuclear RNA was isolated with RNazol. cDNA was prepared by denaturing 5 μ g of linear plasmid DNA at 65°C for 30 min in 0.1 M NaOH followed by the addition of $20 \times$ SSPE [$1 \times$ SSPE = 0.15 M NaCl/10 mM sodium phosphate (pH 7.4)/1 mM EDTA] to a final concentration of $6 \times$ SSPE. The samples were then applied to nitrocellulose filters using a slot-blot apparatus. After UV cross-linking, filters were prehybridized at 42°C for at least 2 h in hybridization buffer [50% (w/v) formamide, $6 \times$ SSPE, $5 \times$ Denhardt's solution, 0.5% (w/v) SDS and 100 μ g/ml salmon sperm DNA] and hybridized at 42°C for three days in fresh hybridization solution containing 10^7 c.p.m. of ^{32}P -labelled transcripts. The filters were then sequentially washed for 30 min in $2 \times$ SSC ($1 \times$ SSC = 0.15 M NaCl/0.015 M sodium citrate)/0.5% (w/v) SDS at 42°C ; in $1 \times$ SSC/0.5% (w/v) SDS at 42°C ; in $1 \times$ SSC/0.5% (w/v) SDS at 65°C and in $0.1 \times$ SSC/0.1% (w/v) SDS at 65°C . The dried filters were then exposed to X-ray films at -70°C with intensifying screens and quantified as described above.

ADRP protein expression by Western-blot analysis

For each experimental condition, 1246 cells cultivated in 35-mm dishes were washed once with PBS and lysed by adding 300 μ l of Laemmli sample buffer [62.5 mM Tris/HCl, pH 6.8/2% (w/v) SDS/10% (v/v) glycerol] but without 2-mercaptoethanol and Bromophenol Blue. Cell lysates were then sonicated, on ice, at 40% of 20 W output for 10 s using a Vibra Cell sonicator and centrifuged at 10000 g for 10 min. The protein concentrations of the cell lysates were determined using a micro bicinchoninic acid (BCA) reagent kit (Pierce, Rockford, IL, U.S.A.). A one tenth volume of $10 \times$ loading dye [50% (v/v) 2-mercaptoethanol/1% (w/v) Bromophenol Blue] was added to samples (20 μ g of protein) before separation by SDS/PAGE (10% acrylamide) according to the method of Laemmli [25], using a minigel apparatus (Bio-Rad Laboratories, Richmond, CA, U.S.A.). Proteins were then electrophoretically transferred to a 0.2 μ m PVDF membrane (Millipore, Bedford, MA, U.S.A.) at 150 mA for 3 h. The membranes were blocked by incubation in TBS [20 mM Tris/HCl (pH 7.5)/500 mM NaCl] containing 3% (w/v) non-fat milk for 2 h at room temperature, incubated for 1 h at room temperature with horseradish peroxidase-conjugated rabbit anti-ADRP IgG [3] in TTBS buffer [TBS buffer containing 0.05% (v/v) Tween 20] containing 0.1% (w/v) BSA and were then washed in TTBS buffer. Immunoreactive proteins on the membranes were revealed by the enhanced chemiluminescence (Amersham). Quantification of ADRP was by densitometric scanning.

RESULTS

Stimulation of ADRP mRNA expression by treatment of adipocyte precursors with cyclooxygenase inhibitors

We have shown previously that treatment of confluent 1246 cells with cyclooxygenase inhibitors for at least 48 h stimulated differentiation up to 18-fold, whereas lipoxygenase inhibitors had no effect [11]. In the present study, we have examined the effect of these compounds on ADRP mRNA expression. Undifferentiated 1246 cells at day 4 were treated for 24 h with various cyclooxygenase and lipoxygenase inhibitors (indomethacin, ibuprofen, naproxen, AA861 and NDGA) at the concentrations shown in the legend to Figure 1, and which were used in the differentiation assay described previously [11]. Cells were harvested and total RNA was collected in order to measure ADRP mRNA expression by Northern-blot analysis. As shown in Figure 1, ADRP mRNA expression was very low in untreated 1246 cells. When the cells were treated for 24 h with the cyclooxygenase inhibitors indomethacin, ibuprofen or naproxen, a 2- to 4-fold increase in the level of ADRP mRNA expressed in the 1246 cells was observed. In contrast, the lipoxygenase inhibitors, AA861 and NDGA, had no effect.

Stimulation of ADRP mRNA expression by ibuprofen is dose and time dependent

Confluent undifferentiated 1246 cells at day 4 were treated for 24 h with increasing doses of ibuprofen from 3×10^{-6} M to 3×10^{-4} M and ADRP mRNA expression was measured by Northern-blot analysis (Figure 2). ADRP mRNA expression was stimulated in a dose-dependent fashion by ibuprofen. A 2-fold induction of ADRP mRNA expression was observed at a concentration of 3×10^{-5} M and, at the highest concentration of ibuprofen assayed (3×10^{-4} M), stimulation of ADRP mRNA expression was 6-fold (± 0.5 S.E.M.) that of the control.

Time-dependent effect of indomethacin and ibuprofen on ADRP mRNA expression

The stimulation of ADRP mRNA expression was also investigated as a function of incubation time. Confluent monolayers of undifferentiated 1246 cells were treated with 3×10^{-4} M of ibuprofen or 3×10^{-5} M indomethacin from 3–72 h. Control cells

Figure 1 Effect of cyclooxygenase and lipoxygenase inhibitors on ADRP mRNA expression

1246 cells were cultivated in DME/F12 medium supplemented with insulin ($10 \mu\text{g/ml}$), transferrin ($10 \mu\text{g/ml}$), and fetuin ($250 \mu\text{g/ml}$). At day 4, the cells were treated for 24 h with the following cyclooxygenase and lipoxygenase inhibitors: indomethacin or ibuprofen (each at 3×10^{-5} M), naproxen (3×10^{-4} M), AA861 or NDGA (3×10^{-6} M). Higher concentrations of AA861 and NDGA were toxic. Total RNA was isolated by RNeasy and $15 \mu\text{g}$ of total RNA were used to measure ADRP or RPL32 mRNA expression by Northern-blot analysis.

Figure 2 Effect of increasing concentrations of ibuprofen on ADRP mRNA expression

1246 cells were cultivated as described in the Experimental section. On day 4, the cells were treated for 24 h with increasing concentrations of ibuprofen. The ethanol carrier only was added to control cells (Cont). RNA was then extracted and ADRP and RPL32 mRNA expression was measured by Northern-blot analysis using ^{32}P -labelled ADRP and RPL32 cDNA as probes.

were maintained in culture medium without ibuprofen or indomethacin. RNA was extracted at the indicated time points to examine ADRP mRNA expression by Northern-blot analysis. The data were analysed by densitometric scanning and normalized to RPL32 (Figure 3, upper panel). Both indomethacin and ibuprofen-treated cells showed a time-dependent increase in ADRP mRNA expression, whereas no change was observed in untreated cells although ibuprofen appeared to be a more potent stimulator of ADRP expression than indomethacin, particularly for short incubation times. ADRP mRNA levels were already increased as early as 3 h after the addition of ibuprofen. A marked increase was observed at 12 h of incubation, both for ibuprofen and for indomethacin. Interestingly, stimulation of ADRP mRNA expression by ibuprofen and indomethacin continued to increase steadily with long-term exposure, even after 72 h. In this case, stimulation over control was 13.3 ± 1.7 -fold for indomethacin and 19 ± 1.8 -fold for ibuprofen (mean $\pm$ S.E.M.) respectively.

We then examined the expression of other markers of differentiation in 1246 cells treated with or without cyclooxygenase inhibitors. In particular, we measured lipoprotein lipase (LPL) expression, which is known to increase in 1246 cells concomitantly with ADRP expression after differentiation has been induced [3]. As shown in Figure 3 (lower panel), no stimulation of LPL mRNA expression was observed for up to 48 h. A small stimulation of LPL mRNA expression was observed at 72 h of exposure, which was probably correlated with a low level of adipose differentiation stimulated by long term exposure of the 1246 cells to ibuprofen only [11]. However, even with this long incubation time, the stimulation of LPL expression by ibuprofen was very small when compared with that observed for ADRP. Similarly, short-term treatment of 1246 cells with ibuprofen failed to stimulate expression of fatty acid binding protein (FAB) mRNA (results not shown). The results indicated that indomethacin and ibuprofen acted specifically on ADRP expression without affecting other markers of differentiation, which are known to be temporally expressed with the same time course as ADRP in differentiated cells.

Figure 3 Time course of indomethacin and ibuprofen effects on ADRP mRNA expression

1246 cells were cultivated in defined medium as described in the Experimental section. On day 4, cells were treated with 3×10^{-5} M indomethacin or 3×10^{-4} M ibuprofen. Control cells were treated with the same volume of ethanol only. Total RNA was extracted at the indicated times. ADRP mRNA expression was measured by Northern-blot analysis. Upper panel: data obtained by densitometric scanning were normalized to the expression of RPL32, which was used as an internal standard. The values are expressed as the ratio of stimulation (mean $\pm$ S.E.M.) in treated cells and ADRP mRNA expression in untreated cells. Lower panel: 1246 cells were treated on day 4 with 3×10^{-4} M ibuprofen and total RNA was collected at time points between 0 and 72 h. ADRP and LPL mRNA expression was measured by Northern-blot analysis.

Transcriptional activation of the ADRP gene by indomethacin and ibuprofen

The data presented above showed that ADRP mRNA expression was stimulated by ibuprofen and indomethacin in a time- and dose-dependent fashion in undifferentiated 1246 cells. Stimulation was observed as early as 3–6 h and was prolonged until 72 h. These data would suggest that the stimulatory effect of ibuprofen may be due to either transcriptional activation or post-

Figure 4 Transcriptional activation of the ADRP gene by indomethacin and ibuprofen

1246 cells were cultivated to confluence in defined medium. They were then switched to fresh defined medium alone (control) or medium containing either 3×10^{-5} M indomethacin (Indo) or 3×10^{-4} M ibuprofen (Ibup) for an additional 5 h. Nuclei were isolated and nuclear transcription assays were performed as described in the Experimental section. Labelled RNA (10^7 c.p.m.) was then hybridized for 3 days with 5 μ g of cDNA for ADRP, FAB, actin and SK plasmid (used as negative control) immobilized to nylon filters. The filters were washed and subjected to autoradiography as described in the Experimental section.

transcriptional stabilization of the ADRP message. To address the issue of transcriptional activation, we performed nuclear run-on experiments on nuclei isolated from 1246 cells treated for 5 h with 3×10^{-5} M of indomethacin or 3×10^{-4} M of ibuprofen. Control cells were maintained in the absence of cyclooxygenase inhibitors for the same length of time. Nuclear-transcription assays were carried out as described in the Experimental section. Labelled RNA was then hybridized with 5 μ g of cDNAs for ADRP. Hybridization to SK plasmid, actin and FAB cDNA was measured as internal controls. As shown in Figure 4, transcription of ADRP was increased by 2.5-fold and 3.5-fold, respectively, in indomethacin- and ibuprofen-treated cells when compared with control cells. Neither compound activated the transcription of FAB.

ADRP mRNA stability is not affected by ibuprofen treatment

To evaluate the possibility that increased mRNA stability may also contribute to the accumulation of ADRP mRNA upon ibuprofen and indomethacin treatment, experiments were carried out to determine the half life of ADRP message in cells treated with or without ibuprofen. Confluent 1246 cells were untreated or treated with 3×10^{-4} M ibuprofen followed by the addition of actinomycin D (5 μ g/ml) in order to block transcriptional activity. RNA was extracted at various time points and ADRP mRNA levels were measured. As shown in Figure 5, the estimated half life of ADRP message was about 5 h both in ibuprofen treated and in control cells. This result suggests that ibuprofen treatment did not alter ADRP message stability and that the accumulation of ADRP mRNA after ibuprofen treatment is most probably due to activation of ADRP gene transcription.

Time-dependent induction of ADRP protein expression by indomethacin and ibuprofen

Experiments were carried out to investigate the status of ADRP protein expression in cells treated with ibuprofen and indomethacin by Western-blot analysis as described in the Ex-

Figure 5 Effect of ibuprofen on ADRP mRNA stability

1246 cells, cultivated in defined medium, were treated on day 4 for 48 h with 3×10^{-4} M ibuprofen (Ibup) or with ethanol alone as control (CTRL). At the end of the incubation period, cells were treated with $5 \mu\text{g/ml}$ of actinomycin D for the times indicated. Total RNA was isolated and ADRP mRNA expression measured by Northern-blot analysis. (A) results of the autoradiograph. (B) Results of densitometric analysis of ADRP mRNA expression normalized to that of RPL32 and plotted as the percentage of ADRP mRNA expression before treatment with actinomycin D (100% at $t = 0$).

Figure 6 Time-dependent stimulation of ADRP protein expression by indomethacin and ibuprofen treatment

1246 cells were grown to confluence and treated with 3×10^{-5} M indomethacin (upper panel) or 3×10^{-4} M ibuprofen (lower panel). Whole cell lysates ($20 \mu\text{g}$) were collected at the indicated time points and were analysed by Western-blot analysis using affinity-purified horseradish-peroxidase-conjugated anti-ADRP antibody. Signals were visualized by enhanced chemiluminescence.

perimental section (Figure 6). As was observed for ADRP mRNA expression, the level of the 50 kDa ADRP protein was low in untreated cells and increased in a time-dependent fashion after treatment with either ibuprofen or indomethacin. At 72 h, the stimulation of ADRP protein expression was 7 ± 0.6 -fold for ibuprofen and 6 ± 0.7 -fold for indomethacin (means $\pm$ S.E.M.).

ADRP level is also regulated by cyclooxygenase inhibitors in adipocytes

Since ADRP expression increases as the cells differentiate into adipocytes [3], experiments were carried out to investigate

Figure 7 Effect of ibuprofen on ADRP mRNA and protein expression in differentiated 1246 cells

1246 cells were differentiated into adipocytes in defined medium as described previously [11]. On day 12, the cells were maintained for 24 h in serum-free medium followed by treatment for 24 h with the concentrations of ibuprofen indicated. Total RNA and cell lysates were used to measure ADRP mRNA and protein expression. Upper panel: ADRP mRNA expression was measured by Northern-blot analysis of total RNA extracted from the cells after 24 h treatment with ibuprofen. Lower panel: ADRP protein expression was measured by Western-blot analysis using $20 \mu\text{g}$ of cell lysates and horseradish-peroxidase-conjugated affinity purified anti-ADRP antibody.

whether ibuprofen had any effect on ADRP expression in differentiated 1246 cells. Fully differentiated 1246 adipocytes at day 12 were maintained in DME/F12 medium and treated for 24 h with increasing concentrations of ibuprofen. Expression of ADRP mRNA was then examined by Northern-blot analysis. As shown in Figure 7 (upper panel), ibuprofen treatment of the differentiated cells increased ADRP mRNA expression in a dose-dependent fashion. A 7.5 ± 0.4 fold stimulation (means $\pm$ S.E.M.) was observed with a concentration of 3×10^{-4} M ibuprofen. Western-blot analysis with anti-ADRP antibodies indicated that ibuprofen also increased the level of ADRP protein in differentiated cells (Figure 7, lower panel). Similarly, indomethacin also stimulated ADRP mRNA and protein expression in differentiated adipocytes (results not shown).

DISCUSSION

The data presented here demonstrate that expression of ADRP mRNA and protein is stimulated by the cyclooxygenase inhibitors ibuprofen, indomethacin and naproxen in adipocyte precursors and in adipocytes, using the adipogenic cell line 1246 as a model system. The stimulatory effect of indomethacin on ADRP expression in confluent undifferentiated cells was also observed

with another adipogenic cell line 3T3-L1, indicating that it is not restricted to the 1246 cell line (X. Xia and G. Serrero, unpublished work). Stimulation of ADRP mRNA and protein expression by ibuprofen and indomethacin was time- and dose-dependent. Nuclear run-on experiments indicated that stimulation of ADRP expression in undifferentiated cells by cyclooxygenase inhibitors occurred at the transcriptional level. Treatment with cyclooxygenase inhibitors did not affect the half life of ADRP mRNA. The short-term stimulation of ADRP expression by cyclooxygenase inhibitors was not due simply to stimulation of differentiation, since other early markers of adipose differentiation, such as LPL, did not increase until 2–3 days of exposure, probably because the differentiation programme had, by then, been initiated. Since the 1246 cells were cultivated in defined medium containing insulin and fetuin, we also examined the effect of these factors on the expression of ADRP in adipocyte precursors. Insulin and fetuin did not stimulate the expression of ADRP and they did not potentiate the stimulatory effect of ibuprofen on ADRP expression (G. Serrero and N. M. Lepak, unpublished work; C. Chen and G. Serrero, unpublished work).

It should be noted that the stimulation of ADRP mRNA expression by ibuprofen and indomethacin was achieved only after cells had reached confluency. Addition of the cyclooxygenase inhibitor at day 3, when the cells were still proliferating, had no effect on ADRP expression (results not shown). The results suggest that induction of ADRP mRNA by these compounds require cell–cell contact or growth arrest. Williams et al. [26] have reported that glucocorticoid induction of the adipocyte clone 5 gene in TA1 cells also required high cell density. We have shown previously that adipose differentiation of the 1246 cell line was stimulated by exposure to cyclooxygenase inhibitors [11]. The results presented here indicate that ADRP is one of the targets of the effect of cyclooxygenase inhibitors on adipose differentiation. One interesting problem is to determine the mechanism of action by which cyclooxygenase inhibitors stimulate ADRP expression. We have shown previously that PGF 2α and 9 α ,11 β -PGF 2α are potent adipose-differentiation inhibitors for adipogenic cell lines and for adipocyte precursors in primary culture [10–12] mediated by activation of prostaglandin F 2α FP receptors [27]. Short-term exposure of the 1246 cells to PGF 2α and to arachidonic acid at the time of differentiation resulted in inhibition of ADRP expression (H. Ye and G. Serrero, unpublished work). These data point to an inhibitory role for arachidonate metabolites on ADRP expression. However, for several reasons, it is difficult to conclude that the stimulatory effect of indomethacin on ADRP expression may only be mediated by inhibiting prostaglandin production. Since ADRP is increased during the adipose differentiation process, one could assume that the inhibition of ADRP expression by PGF 2α could be associated with the inhibitory effect on the adipose differentiation process itself and not with a direct effect of PGF 2α on ADRP expression. Secondly, the concentration of indomethacin required to stimulate ADRP expression is higher than the concentration normally required to inhibit prostaglandin synthesis in adipogenic cell lines [16]. It has been shown recently that the peroxisome proliferator receptor, PPAR γ , is activated by indomethacin and other non-steroidal anti-inflammatory drugs in the C3H 10T1/2 cell line [19]. Indomethacin was found to bind directly to the PPAR γ receptor and to act as a PPAR γ agonist, suggesting that this is the way by which indomethacin stimulates adipose differentiation [19]. Based on these results, it is possible to assume that the effect of indomethacin or ibuprofen on ADRP expression could be secondary to the prior activation of PPAR γ rather than a direct effect. Peroxisome proliferator response elements (PPREs) have

been identified in a number of adipocyte genes transcriptionally activated during adipocyte differentiation [20,28–30] upon activation of PPAR γ . However, analysis of the ADRP promoter failed to identify the presence of PPREs (B. Condon, J. Gao and G. Serrero, unpublished work). Moreover, other potent activators of PPAR γ , such as clofibrate, icosatetraenoic acid and 9-*cis*-retinoic acid were not as potent stimulators of ADRP expression, unlike cyclooxygenase inhibitors.

It should be noted that in addition to stimulating ADRP expression in undifferentiated cells, indomethacin and ibuprofen also stimulated ADRP mRNA and protein expression in fully differentiated adipocytes. This would suggest the importance of ADRP and of its regulation by cyclooxygenase inhibitors in mature adipocytes. The fact that the stimulatory effect of ibuprofen on ADRP expression was inhibited by treatment with 5 μ g/ml of actinomycin D (results not shown) suggests that, in adipocytes, ibuprofen stimulates ADRP expression at the transcriptional level, similarly to that observed for adipocyte precursors (Figure 4).

The data presented here provide new information about the regulation of ADRP, an early, inducible gene of the adipocyte differentiation programme and identify an additional and novel target for the action of cyclooxygenase inhibitors during adipocyte differentiation and in mature adipocytes.

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