Control of terminal differentiation of adipose precursor cells by glucocorticoids

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Abstract The role of glucocorticoids on adipose conversion has been studied using confluent Ob1771 mouse preadipose cells maintained in a serum-free culture medium able to support the emergence of early but not that of late markers of differentiation. Under these culture conditions, glucocorticoids play, at physiological concentrations, a permissive role for terminal differentiation, characterized by glycerol-3-phosphate dehydrogenase expression and triacylglycerol accumulation within 12 days, whereas progesterone, testosterone, and estradiol are inactive. Glucocorticoids behave as mitogenic-adipogenic stimuli able to trigger growth-arrested, early marker-expressing cells to enter the terminal phase of the differentiation program and thus appear to mimic the mitogenic-adipogenic activity already described for arachidonic acid and cyclic AMP-elevating agents, especially prostacyclin. When compared to corticosterone alone, exposure of Ob1771 cells to both corticosterone and arachidonic acid leads to an additional increase in the glycerol-3-phosphate dehydrogenase activity and number of differentiated cells; this potentiation is further enhanced when the culture medium is supplemented with the cyclic AMP phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine. This suggests indirectly the involvement of prostacyclin as a metabolite of arachidonic acid able to induce cyclic AMP accumulation. In agreement with this hypothesis, it is found that a promoting effect is exerted by corticosterone on the metabolism of arachidonic acid, leading in turn to an increase in the production of prostacyclin. These findings allow a better understanding of the role of glucocorticoids on adipose cell differentiation and explain a posteriori the effectiveness of the combination of dexamethasone-isobutylmethylxanthine used in innumerable studies.—**Gaillard, D., M. Wabitsch, B. Pipy, and R. Négrel.** Control of terminal differentiation of adipose precursor cells by glucocorticoids. *J. Lipid Res.* 1991. **32:** 569–579.

Supplementary key words corticosterone • dexamethasone • adipose conversion • differentiation • preadipose cells • preadipocytes • arachidonic acid • prostanoids • prostacyclin

Cells from various preadipocyte clonal lines, able to differentiate in vitro, have allowed new insights into the events that occur during the process of adipose conversion (1). The expression of early markers such as lipoprotein lipase and pOb_{24} mRNA in Ob1771 preadipocytes takes place at growth arrest (2, 3). In contrast, a limited proliferation of these already committed, early marker-containing cells, is subsequently required for the expression of late markers of adipose conversion such as glycerol-3-phosphate dehydrogenase (GPDH) and triacylglycerol accumulation (4–6). This situation, which suggests that terminal differentiation of adipose cells is controlled by specific mitogenic stimuli, is in agreement with in vivo data obtained by means of pulse-labeling of [^{3}H]thymidine into DNA during the development of adipose tissue in newborn rodents (7, 8).

The establishment of serum-free hormone-supplemented media, able to support growth and/or differentiation of preadipocyte cell lines or cell strains (9–13), has provided the opportunity to characterize some extracellular and intracellular signals that are involved in the control of adipose conversion (14, 15). Using this approach, we have previously shown that cells of the Ob1771 preadipocyte clonal line, able to express lipoprotein lipase in 5F medium (containing insulin, transferrin, triiodothyronine, growth hormone, and fetuin), required additional mitogenic stimuli to enter the terminal part of the differentiation program (14, 15). Among these stimuli, agents able to trigger the cyclic AMP pathway played a pivotal role, whereas those able to trigger inositol phospholipid breakdown were acting as modulators. Arachidonic acid, as a promoter of both the cyclic AMP and the inositol phospholipid pathways via two of its metabolites, prostacyclin (PGI_2) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), respectively,

Abbreviations: GPDH, glycerol-3-phosphate dehydrogenase; PGI_2, prostacyclin; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; DME medium, Dulbecco's modified Eagle's medium; FGF, fibroblast growth factor; T_3, triiodothyronine; LDH, lactate dehydrogenase; EGF, epidermal growth factor; IBMX, 3-isobutyl-1-methylxanthine; TLC, thin-layer chromatography; HPLC, high performance liquid chromatography.

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was indeed able to trigger terminal differentiation when added to 5F medium (14, 15).

The role of glucocorticoids has been the subject of numerous investigations both in vivo and in vitro. Glucocorticoids, which are thought to be key hormones for adipose tissue development (16), in particular the abdominal fat depot (17), have been shown to promote in vitro the differentiation of adipose precursor cells from various species (18–23). We have recently reported a promoting effect of glucocorticoids on the differentiation of human adipose precursor cells in a serum-free chemically defined medium (22). However, the mechanisms by which these hormones are active in adipose cell differentiation after binding to the glucocorticoid receptor are still unclear. In the present report we demonstrate that glucocorticoids behave as adipogenic-mitogenic stimuli and are able to trigger the terminal differentiation of mouse Ob1771 preadipocyte cells maintained in 5F medium. In agreement with our previous findings (14, 15), the growth- and differentiation-promoting effect of corticosterone appears to be related to its ability to stimulate arachidonic acid metabolism leading especially to the enhancement of prostacyclin production. The adipogenic-mitogenic effect of glucocorticoids is discussed with respect to adipose conversion in vitro and adipose tissue development in vivo.

MATERIALS AND METHODS

Cell culture

Stock cultures of Ob1771 cells, obtained after subcloning of Ob17 cells, established from the periepididymal adipose tissue of the genetically obese C57B16J mouse (24), were maintained in Dulbecco's modified Eagle's (DME) medium supplemented with biotin, pantothenate, antibiotics, and 10% (v/v) fetal bovine serum as previously described (25). All experiments were performed in serum-free hormone-supplemented medium with cells previously grown in 4F medium consisting of a mixture of DME medium and Ham's F_{12} medium (1:1, v/v) containing insulin (5 μg/ml), transferrin (10 μg/ml), a partially purified kallikrein-like activity from rat submaxillary gland [1 μg/ml, (26)] and fibroblast growth factor (FGF, 25 ng/ml). Confluent cells grown under these latter conditions were subsequently shifted (day zero) to the same basal medium as above enriched with sodium ascorbate (100 μM) plus sodium selenite (20 nM) and containing insulin (5 μg/ml), transferrin (10 μg/ml), fetuin (500 μg/ml), bovine growth hormone (2 nM), and triiodothyronine (T_3, 200 pM). This culture medium, referred to as 5F medium (14, 15), was further supplemented or not as indicated in the Results section. Two medium changes were subsequently performed at days 3 and 7. All added compounds, dissolved in ethanol, were present in the culture medium as a 1:100 dilution of the stock solution and thus ethanol concentrations never exceeded 1%. Control experiments showed that exposing the cells to 1% ethanol had no effect on the various parameters under investigation. Primary cultures of adipose precursor cells present in the stromal-vascular fraction of mouse or human adipose tissues were obtained after collagenase digestion. After plating in serum-supplemented medium and careful washings, these cultures were maintained under the same serum-free culture conditions as Ob1771 cells (12, 13).

Biochemical assays

GPDH (EC 1.1.1.8) and lactate dehydrogenase (LDH; EC 1.1.1.27) were assayed spectrophotometrically in cell homogenates as previously described (14, 15). Assays were carried out in duplicate at day 12 after confluence. Inter-assay variability as well as variability among mean values from separate wells maintained under identical culture conditions never exceeded 5% in the same series of cells. Protein and DNA contents of cell homogenates were determined according to Lowry et al. (27) and Labarca and Paigen (28), respectively. GPDH specific activity was expressed in milliunits (nmoles/min) per mg of protein.

Intracellular cyclic AMP concentrations and inositol phospholipid breakdown were measured as previously reported (14, 15) using a protein binding assay and [^{3}H]inositol-prelabeled cells, respectively.

The epidermal growth factor (EGF) receptor-binding assay used to monitor the in vivo activation of protein kinase C was performed as described by Doglio et al. (29).

Metabolism of arachidonic acid in Ob1771 cells

Confluent cells were labeled with [^{3}H]arachidonic acid (10^{-6} M, 0.5 μCi/culture well) in 5F medium for 48 h. Under these conditions 80 ± 5% of the radioactivity was incorporated into the cells, 95% of which was incorporated into polar lipids as checked by TLC of the extracted cellular lipids using hexane–diethylether–formic acid 80:20:1 (v/v) as the developing solvent. These prelabeled cells were washed with DME-F_{12} medium 1:1 (v/v) and maintained for the next 24 h in 5F medium (0.5 ml) alone or supplemented as indicated in the text (see also the legend of Fig. 4). Each culture medium was pooled with its respective cell wash (0.5 ml of DME/F_{12} medium) and collected in the presence of butyl-hydroxytoluene (50 μM). Labeled arachidonic acid and the metabolites released into the culture medium were recovered after acidification with HCl either by solvent extraction using a mixture of cyclohexane–ethyl acetate 1:1 (v/v) or by chromatography on Sep-Pak C_{18} cartridges (Waters) as described by Powell (30). Arachidonic acid metabolites were eluted with methanol (5 ml). After drying under nitrogen, the

extracted material was dissolved in a small volume of methanol-ethyl acetate 1:1 (v/v) and aliquots of known radioactivity content were applied to thin-layer silica gel plates. TLC plates were developed to a height of 19 cm in the organic phase of ethyl acetate-isooctane-acetic acid-water 11:5:2:10 (v/v) with unlabeled or tritiated standards run in parallel. Plates were cut into segments of 1 cm and the radioactivity in each zone was assayed by liquid scintillation counting. HPLC analysis was performed after extraction of samples using Sep-Pak C_{18} cartridges as described above. The dry extracted lipids were suspended in acetonitrile-water-acetic acid 35:65:0.6 and subjected to reverse-phase HPLC. A Hewlett-Packard HPLC system was used with a Hewlett-Packard 5-μm C_{18} column (200 × 4 mm). The solvent, acetonitrile-water-acetic acid buffered to pH 5.0 with triethylamine, was used at a 1.1 ml/min flow rate. Arachidonic acid metabolites were eluted during an initial isocratic phase for 7 min (35:65:0.6), a stepwise gradient increase of acetonitrile to 91:9:0.6 for 37 min, and during a final isocratic phase for 10 min (91:9:0.6). The radioactivity in the effluent was monitored with a Berthold LB506D model HPLC radioactivity detector.

Materials

Steroids were purchased from Sigma Chemical Co. (St. Louis, MO), [^{3}H]arachidonic acid from Dupont New England Nuclear (Paris, France), and ^{125}I-labeled EGF from Amersham International (Amersham, Bucks, U.K.). The sources of culture reagents and all other products were the same as reported previously (14, 15, 22).

RESULTS

Adipogenic-mitogenic effect of glucocorticoids

Confluent Ob1771 cells, maintained in 5F medium, are known to express early markers of differentiation such as lipoprotein lipase (31) and pOb_{24} mRNA [(32) and A. Pradines-Figuères, D. Gaillard, and R. Négrel, unpublished results]. Addition to 5F medium of physiological concentrations (10^{-9} M to 10^{-7} M) of corticosterone, the major circulating glucocorticoid in rodents, allowed the cells to express late markers indicative of terminal differentiation. Induction of terminal differentiation by corticosterone is illustrated by the expression of high levels of GPDH activity used as an indicator of terminal differentiation measured after 12 days of exposure to the hormone (**Fig. 1**). Induction of terminal differentiation is also illustrated by the cytoplasmic accumulation of triacylglycerol, easily detectable under the microscope as refringent lipid droplets in a variable number of cells as shown in the photomicrographs of **Fig. 2.** It should be

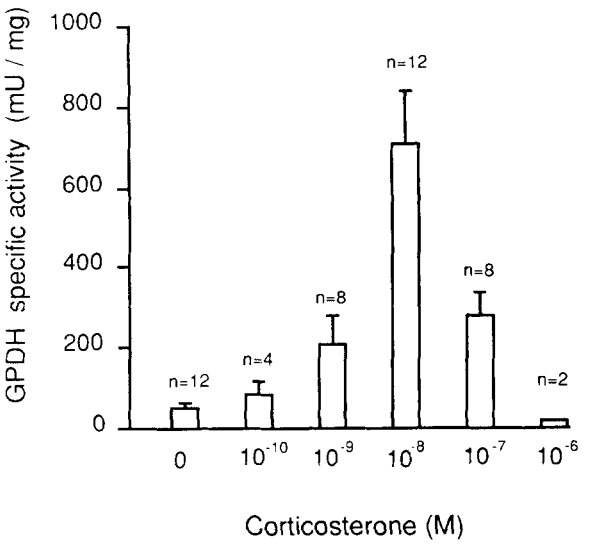

Fig. 1. Effect of corticosterone on the activity levels of glycerol-3-phosphate dehydrogenase in Ob1771 cells. Confluent Ob1771 cells were maintained for 12 days in 5F medium supplemented or not with various concentrations of corticosterone as indicated. GPDH activities were then determined as described in Materials and Methods. The values are the means ± SEM of duplicate determinations obtained from 4 to 12 (n) independent series of cells.

recalled that a tight correlation has been previously observed between the specific activity of GPDH measured at day 12 and the proportion of triacylglycerol-containing cells under various serum-free culture conditions (14). The adipogenic effect of corticosterone in 5F medium was dose-dependent and culminated very reproducibly at 10^{-8} M for both GPDH activity (Fig. 1) and the number of triacylglycerol-containing cells (Fig. 2, A-E). Long-term treatments with higher concentrations of corticosterone were less effective (10^{-7} M) or even became inhibitory (10^{-6} M). Similarly, the maximally effective adipogenic concentration of corticosterone was found to be 10^{-8} M for primary culture of mouse adipose precursor cells present in the stromal vascular fraction isolated from subcutaneous or periepididymal adipose tissue (Fig. 2F).

The effects of chronic exposure of Ob1771 cells to other steroids on the expression of GPDH are shown in **Fig. 3.** Hydrocortisone had effectiveness and potentiality similar to corticosterone, whereas cortisone behaved as a poor adipogenic agent. Dexamethasone was the most potent steroid: its adipogenic activity was already significant at 10^{-10} M. In contrast, aldosterone and progesterone were less potent since concentrations of 10^{-7} M or even 10^{-6} M were required to promote GPDH expression. Testosterone, estrone, 17α- and 17β-estradiol were found to be inactive when added to 5F medium at concentrations ranging from 10^{-10} to 10^{-6} M. According to *i*) the binding properties of the glucocorticoid receptor, which has been described in various tissues (33), including adipose tissue

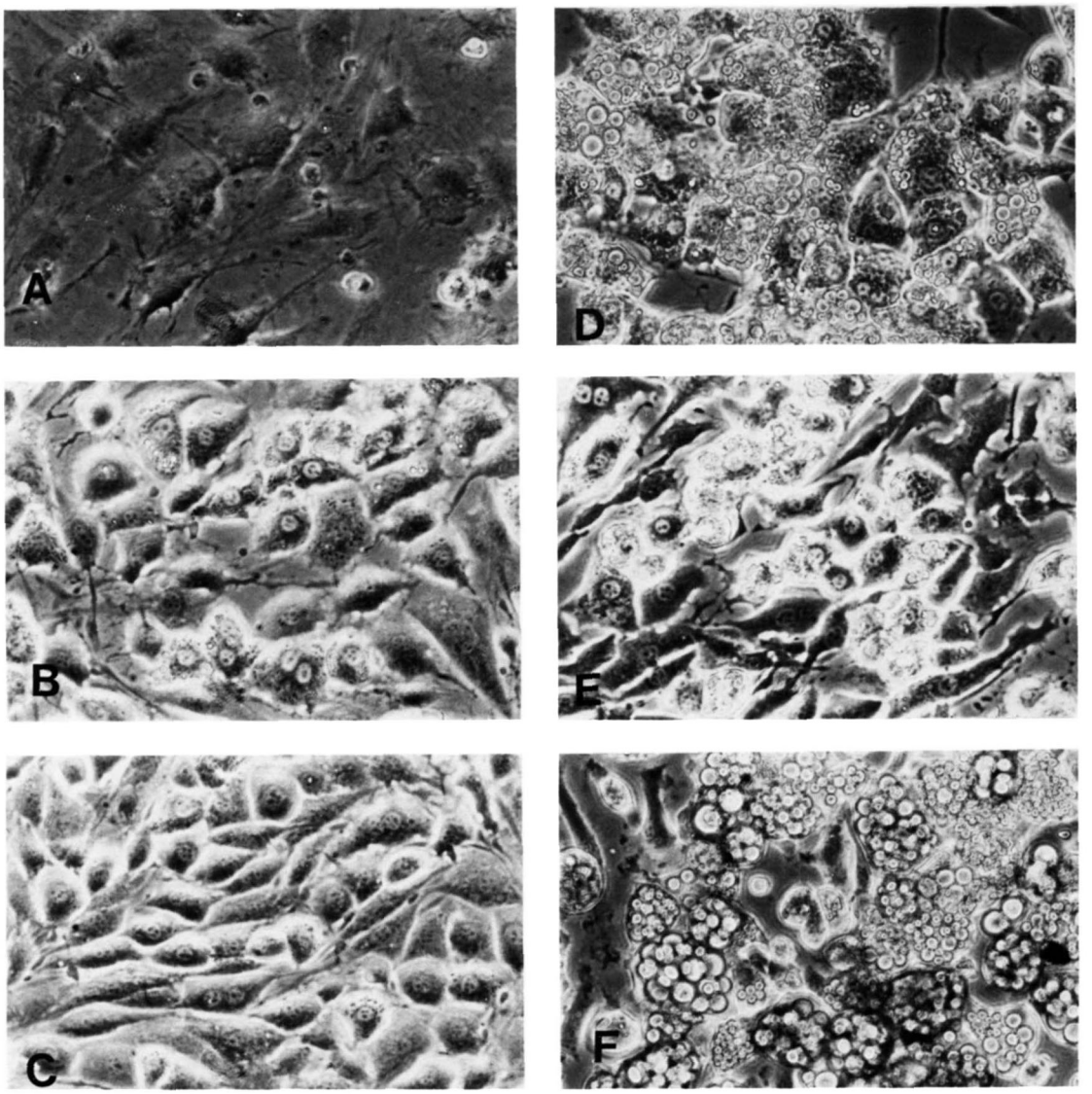

Fig. 2. Morphological differentiation of Ob1771 cells and mouse adipose precursor cells exposed to corticosterone in serum-free hormone supplemented medium. Photomicrographs (magnification ×250) of representative fields of Ob1771 cells (A–E) and stromal-vascular cells from adult mouse subcutaneous adipose tissue (F) were taken after 6 (A–C) or 12 (D–F) days of exposure to 5F medium alone (A) or 5F medium supplemented with either 10^{-8} M (B, D, and F) or 10^{-7} M corticosterone (C and E). Ob1771 cells were previously grown during the exponential phase as described in Materials and Methods. Primary cultures of mouse stromal vascular cells were shifted to the serum-free culture conditions after plating overnight in serum-supplemented medium as previously described for rat and human cells (12, 13).

(34, 35), and *ii*) the order of potency of the various steroids, these results indicate that the adipogenic effect of steroid hormones in Ob1771 cells was specific for glucocorticoids.

It is noteworthy that, whatever the active steroid being used as an agonist, a strong reduction in its adipogenic activity was systematically observed beyond the maximally effective concentration. For instance, long-term treatment with the most potent glucocorticoid, dexamethasone, which is maximally active at 10^{-9} M did not lead to an increase in GPDH activity when present at 10^{-8} M (Fig. 3). The loss of this promoting effect at high concentrations of the hormones on GPDH activity was accompanied by a parallel lack of the intracellular lipid accumulation (Fig. 2E).

The effects of increasing concentrations of corticosterone were also investigated when Ob1771 cells were exposed to the hormone only for the first 3 days after confluence with assays being performed at day 12 (**Table 1**). Under these conditions, the adipogenic effect of corticosterone was less pronounced but the inhibitory effect observed at 10^{-7} M during chronic exposures was no longer apparent. Interestingly, a potent enhancement, leading to a dramatic increase in the GPDH activity at day 12, was obtained when 5F medium was also supplemented during the first 3 days

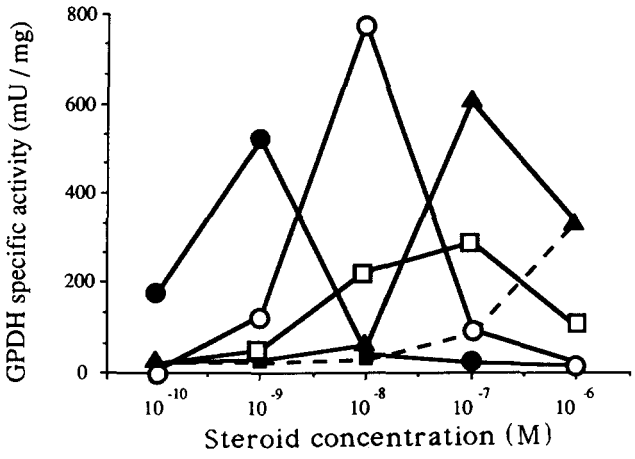

Fig. 3. Adipogenic effect of glucocorticoids in Ob1771 cells. GPDH activities were determined in confluent cells maintained for 12 days in 5F medium supplemented with the indicated concentrations of dexamethasone (●), hydrocortisone (○), cortisone (□), d-aldosterone (▲), and progesterone (■). The values are the mean of duplicate determinations obtained in two independent series of cells; they did not differ by more than 10%. Testosterone, estrone, 17α- and 17β-estradiol tested from 10^{-10} M to 10^{-6} M failed to induce GPDH expression and triacylglycerol accumulation.

with 10^{-4} M isobutyl methylxanthine (IBMX) as a cyclic AMP phosphodiesterase inhibitor (Table 1). A similar result was obtained when confluent Ob1771 cells were exposed for the first 3 days to IBMX and to the stable analog of prostacyclin, i.e., carbaprostacyclin (cPGI$_2$), added as an activator of cyclic AMP production (Table 1 and ref. 15).

Since terminal differentiation of growth-arrested, early marker-containing Ob1771 cells in 5F medium is dependent on DNA synthesis (2–6) and is preceded by at least one round of cell division (14), adipogenic glucocorticoids should also behave as mitogenic stimuli. As expected, exposure of confluent Ob1771 cells to 10^{-8} M or 10^{-7} M corticosterone led to increases in cell number that were readily apparent under the microscope within 3 to 6 days (Fig. 2, A–C). At day 12, when differentiated cells were harvested for GPDH determination, the DNA content of both 10^{-8} M and 10^{-7} M corticosterone-treated cultures was threefold higher than in control cultures: 1.52 ± 0.12 versus 0.51 ± 0.06 μg DNA/culture well. Likewise, a dose-dependent increase in DNA content at day 12 was observed when Ob1771 cells were exposed for the first 3 days after confluence to increasing concentrations of corticosterone providing the simultaneous presence of IBMX (Table 1). In contrast to glucocorticoids, but in agreement with their lack of adipogenic activity, testosterone, estrone, and 17α- and 17β-estradiol failed to promote a cell increase within 12 days when present in 5F medium at concentrations ranging from 10^{-10} M to 10^{-6} M (data not shown).

Relationships between corticosterone, cyclic AMP, and arachidonic acid metabolism

In the light of our previous findings using Ob1771 cells grown and maintained in serum-free medium (14, 15), the mitogenic-adipogenic effect of glucocorticoids should be linked to a stimulation of the intracellular events involved in the control of the critical mitoses which lead to terminal differentiation. In that respect the cyclic AMP pathway, which is the key intracellular signal for this process in Ob1771 cells, appeared as an event that might be affected by these hormones. A possible relationship between glucocorticoids and the cyclic AMP pathway in triggering

TABLE 1. Adipogenic-mitogenic effect of corticosterone during a 3-day exposure in the presence or absence of IBMX

Culture Conditions	IBMX	Specific Activity of GPDH	DNA Content
		mU/mg	*µg/culture well*
5F medium	–	40 ± 10	0.46 ± 0.05
5F medium	+	200 ± 30	0.48 ± 0.06
+ Corticosterone 10^{-9} M	–	110 ± 15	0.50 ± 0.05
+ Corticosterone 10^{-9} M	+	470 ± 40	0.60 ± 0.06
+ Corticosterone 10^{-8} M	–	220 ± 20	0.52 ± 0.06
+ Corticosterone 10^{-8} M	+	950 ± 50	0.95 ± 0.07
+ Corticosterone 10^{-7} M	–	250 ± 30	0.55 ± 0.06
+ Corticosterone 10^{-7} M	+	1680 ± 95	1.35 ± 0.08
+ cPGI$_2$ 2 × 10^{-7} M	–	330 ± 35	0.61 ± 0.04
+ cPGI$_2$ 2 × 10^{-7} M	+	1630 ± 80	1.42 ± 0.07

Confluent Ob1771 cells were maintained during the first 3 days in 5F medium supplemented (+) or not (–) with 10^{-4} M IBMX and various concentrations of corticosterone or 2 × 10^{-7} M carbaprostacyclin. At days 3 and 7, cells were exposed to fresh 5F medium without any other supplement and maintained until day 12. Cells were then washed, scraped, and homogenized for GPDH activity, protein, and DNA contents. The activity values are reported as medians ± ranges of duplicate determinations performed with two independent series of cells under each culture condition.

the terminal differentiation was indeed already suggested by the potentiating effect of IBMX on the mitogenic-adipogenic activity of corticosterone (Table 1). However, in contrast to arachidonic acid, $cPGI_2$, or other cyclic AMP-elevating agents, a short-term exposure (5 min) of Ob1771 cells to corticosterone (10^{-10} M to 10^{-7} M) in 5F medium did not lead to any increase in the intracellular content of cyclic AMP (data not shown). Moreover, among other possible intracellular events able to modulate the mitogenic response of Ob1771 cells during terminal differentiation, neither inositol phospholipid breakdown nor protein kinase C activation (monitored through the reduction of ^{125}I-labeled EGF binding) appeared to be affected during short-term exposures (15–30 min) to corticosterone (data not shown). Since none of these short-term intracellular events seemed to be responsible for the effect of glucocorticoids on adipose cell differentiation, possible long-term effects of these hormones were investigated.

According to the model that we have recently proposed (15), cyclic AMP production and protein kinase C activation could be triggered in an autocrine manner by PGI_2 (prostacyclin) and $PGF_{2\alpha}$, respectively, which are both metabolites of arachidonic acid in preadipocyte cells (36–38). Therefore, the influence of corticosterone on terminal differentiation in the simultaneous presence of arachidonic acid was examined. As shown in **Table 2,** corticosterone and arachidonic acid, when present for 12 days in 5F medium, were able to potentiate each other in their ability to promote GPDH expression. The effect of suboptimal concentrations of arachidonic acid (2 and 5 × 10^{-6} M) in inducing GPDH expression was strongly enhanced in the presence of 10^{-9} M and 10^{-8} M corticosterone. Furthermore, when 5F medium was supplemented with 7.5 × 10^{-6} M arachidonic acid, a maximal expression of GPDH (2500 mU/mg), accompanied by accumulation of triacylglycerol in most of the cells, was reproducibly observed at 10^{-9} M corticosterone. This concentration of corticosterone is one order of magnitude lower than the maximally effective concentration of the hormone in the absence of exogenous arachidonic acid. The inhibitory effect of a long-term treatment with 10^{-7} M corticosterone was again readily observed when Ob1771 cells were simultaneously exposed to arachidonic acid or carbaprostacyclin, agents which are able, when present alone, to trigger the differentiation of a very large proportion of the cells. The effect of corticosterone on the expression of GPDH at day 12 was also enhanced during a 3-day exposure of the cells to the hormone in the presence of IBMX and arachidonic acid (Table 2). Under these conditions, the maximally active concentration of corticosterone was shifted from 10^{-7} M to 10^{-8} M.

Altogether, the synergistic effects of arachidonic acid and corticosterone suggested that the mitogenic-adipogenic activity of the hormone might be exerted through the enhanced generation of some metabolite(s) of arachidonic acid, especially able to elicit cyclic AMP production, i.e., prostacyclin. Therefore, [^{3}H]arachidonic acid-prelabeled Ob1771 cells were used to examine the effect of corticosterone on arachidonic acid metabolism.

The results of **Fig. 4** were obtained with Ob1771 cells that had been prelabeled with [^{3}H]arachidonic acid. Cells were then exposed for 24 h to corticosterone and/or exogenous unlabeled arachidonic acid, in the absence or the

TABLE 2. Mutual potentiation of corticosterone and arachidonic acid as adipogenic agents

Culture Conditions	Concentration of Corticosterone			
	0	10^{-9} M	10^{-8} M	10^{-7} M
	GPDH specific activity (mU/mg)			
1) 12-Day exposure				
5F medium	40 ± 10	250 ± 80	500 ± 50	60 ± 15
+ Arachidonic acid (2 × 10^{-6} M)	100 ± 30	700 ± 100	1050 ± 200	200 ± 30
+ Arachidonic acid (5 × 10^{-6} M)	900 ± 80	1560 ± 150	1850 ± 200	380 ± 50
+ Arachidonic acid (7.5 × 10^{-6} M)	1750 ± 200	2500 ± 300	2100 ± 200	200 ± 40
+ $cPGI_2$ (0.2 × 10^{-6} M)	2500 ± 300	2700 ± 250	2300 ± 200	800 ± 70
2) 3-Day exposure				
5F medium + IBMX (1 × 10^{-4} M)	200 ± 30	470 ± 40	950 ± 50	1680 ± 95
+ Arachidonic acid (1 × 10^{-6} M)	300 ± 50	600 ± 70	1500 ± 150	1400 ± 150
+ Arachidonic acid (5 × 10^{-6} M)	800 ± 100	1300 ± 100	2000 ± 190	1750 ± 130

Confluent Ob1771 cells were maintained under the various culture conditions indicated in the left column, in the absence or presence of increasing concentrations of corticosterone. In the first culture protocol (12-day exposure) each culture medium added at day zero was renewed at days 3 and 7. In the second protocol (3-day exposure) cells were maintained under the various indicated culture conditions during the first 3 days only and were fed at days 3 and 7 with 5F medium. In each case, cell homogenates were prepared at day 12 for GPDH and protein determination. The values are the medians ± ranges of duplicate determinations performed with three (12-day exposure) or two (3-day exposure) independent series of cells under each culture condition.

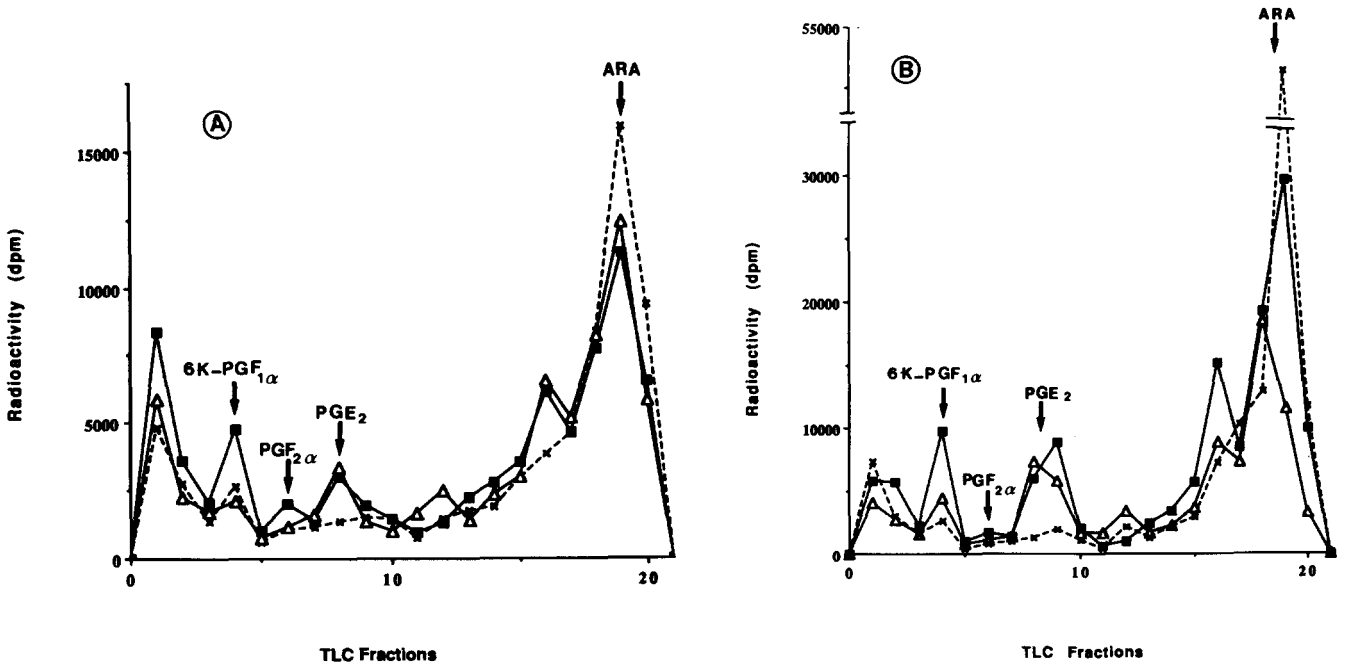

Fig. 4. Effect of corticosterone on the synthesis of prostacyclin in [^{3}H]arachidonic acid-prelabeled cells. Confluent Ob1771 cells prelabeled with [^{3}H]arachidonic acid in 5F medium for 48 h (see Materials and Methods) were washed with DME/F_{12} medium (1:1, v/v) and maintained for the next 24 h in 5F medium alone (△) in Fig. A or enriched with 5×10^{-6} M arachidonic acid (△) in Fig. B. These media were supplemented in Fig. A: with 10^{-8} M corticosterone (■) alone or together with 10^{-3} M aspirin (X) and in Fig. B: with 10^{-9} M corticosterone (■) alone or together with 10^{-3} M aspirin (X). Culture media from six individual culture wells under each culture condition were mixed with their respective cell washes in the presence of butylhydroxytoluene (50 μM). After counting of an aliquot, the collected media from each culture condition were pooled, acidified, extracted with cyclohexane ethyl acetate, and analyzed by TLC as described in Materials and Methods. The radioactivity recovered in each fraction expressed in dpm corresponds to the material present in the lipid extract from six pooled culture wells. The chromatograms are representative of three independent experiments performed under each culture condition. In one of them, extraction of arachidonic acid and its metabolites has been performed using Sep-Pak C_{18} cartridges.

presence of aspirin. First, a higher amount of radioactivity was reproducibly recovered in the lipid extract of the culture medium of corticosterone-treated cells as compared with untreated cells (at 10^{-8} M, the increase was 10 ± 2% of the total). This increase was almost exclusively associated with the peak migrating with authentic 6-keto-$PGF_{1\alpha}$, the stable decomposition product of PGI_2. As expected, when the cells were maintained in the presence of aspirin, a cyclooxygenase inhibitor that has been shown to prevent adipose conversion induced by exogenous arachidonic acid (15), the production of 6-keto-$PGF_{1\alpha}$ as well as that of other prostaglandins was abolished whereas a concomitant increase in unmetabolized arachidonic acid was observed (Fig. 4A). When the cells were simultaneously exposed to unlabeled arachidonic acid, the effect of corticosterone on arachidonic acid metabolism became even more striking (note the change in scale in Fig. 4B). Addition of 5×10^{-6} M arachidonic acid to 5F medium led to an increase of 25 ± 5% in the recovered radioactivity from the lipid extract; this increase rose to 100 ± 10% when 10^{-9} M corticosterone was simultaneously present. Under the latter condition, the rise in radioactivity of the lipid extract was mainly associated with three peaks: unmetabolized arachidonic acid, 6-keto-$PGF_{1\alpha}$, and metabolites that might include HHT, PGA_2, and PGB_2. As control experiments, using a different technique to identify and quantitate the various metabolites of arachidonic acid, analyses by reverse phase HPLC have shown similar results, i.e., higher amounts of the different metabolites, including 6-keto-$PGF_{1\alpha}$, were indeed determined from the lipid extract of the culture medium of corticosterone-treated cells than in that of untreated cells. It is of interest to observe that, whether or not unlabeled arachidonic acid was included in the culture medium, the presence of corticosterone did not lead to an increase in the production of PGE_2 known to be ineffective in the adipose conversion process (15, 39).

DISCUSSION

Since the first reports (18, 40, 41) showing that a 48-h treatment of confluent 3T3-L1 preadipocyte cells in serum-supplemented medium with a mixture of dexamethasone and IBMX led to an acceleration and amplification of their adipose conversion, a similar adipogenic effect of glucocorticoids and/or IBMX has been reported for several other preadipocyte models (19–23). IBMX is

known to enhance the intracellular level of cyclic AMP by inhibiting both soluble and particulate cyclic AMP phosphodiesterases present in these cells (42). Nevertheless, no clear evidence on the role of glucocorticoids in this process has been given so far, although it has been suggested that these hormones might increase the production of a rate-limiting factor required for fat cell differentiation (18, 20, 43).

In the present study, performed with mouse Ob1771 preadipocyte cells maintained in a chemically defined medium, glucocorticoids behaved within a physiological range of concentrations, as mitogenic-adipogenic stimuli known to be required to trigger terminal events of adipose conversion. Under the simplest culture conditions described herein (i.e., 5F medium), glucocorticoids appeared as absolute requirements for terminal differentiation. As reported in most of the previous studies carried out in serum-supplemented media, glucocorticoids appeared also as modulators and/or accelerators when the culture medium was supplemented with other adipogenic compounds such as IBMX and/or arachidonic acid. Although a growth-promoting effect of dexamethasone has already been mentioned for preadipocytes in a few reports (18, 44, 45), the relationship between the mitogenic effect and the adipogenic activity of this glucocorticoid analog had never been reported to our knowledge. Such a mitogenic-adipogenic property is in agreement with the fact that *i*) the development of adipose cell clusters of various preadipocyte models in vitro proceeds through a limited proliferation of committed precursors (4, 46, 47); *ii*) early markers of differentiation such as lipoprotein lipase and pOb24 mRNA (31, 32) are expressed in growth-arrested Ob1771 cells both in serum-supplemented and 5F medium (3, 11); and *iii*) the expression of late markers such as GPDH activity and triacylglycerol accumulation, indicative of terminal differentiation, requires the exposure of growth-arrested cells to some specific mitogenic stimuli (6, 14, 15). Among these specific stimuli, factors able to increase the intracellular level of cyclic AMP are of utmost importance for Ob1771 as well as 3T3 cells (14, 48) and agents able to trigger inositol phospholipid breakdown or to activate protein kinase C more directly act as potentiators or modulators of the mitogenic response (14). Arachidonic acid and two of its metabolites (PGI_2 and $PGF_{2\alpha}$, agonists of the cyclic AMP and the inositol phospholipid pathway, respectively) have been indeed demonstrated to play a pivotal role in the control of terminal differentiation (14, 15). Since corticosterone elicited enhanced arachidonic acid release and increased synthesis of prostacyclin, which is known to lead in turn to cyclic AMP production and mitoses, it is proposed that prostacyclin is the ultimate mediator of the mitogenic-adipogenic activity of corticosterone.

It is striking to observe that, when exposed to 5F medium enriched with an optimal concentration of corticosterone, such as in serum-supplemented medium, differentiated cells were arranged in clusters of variable size. Supplementation of 5F medium with a combination of corticosterone (10^{-9} M) and arachidonic acid ($2–7.5 \times 10^{-6}$ M), a condition that gives rise to a higher production of prostacyclin, was accompanied by a striking increase in the size of differentiated clusters and led very often to a near complete monolayer of differentiated cells. It is thus very likely that the production of prostacyclin, acting in an autocrine manner and by means of its well known ability to increase cyclic AMP (15, 49, 50), represents the limiting event able to induce mitoses and the progression of preadipocytes into the late part of their differentiation program. This involvement of prostacyclin would explain a posteriori the effectiveness of the adipogenic cocktail "dexamethasone-IBMX," used to amplify and to accelerate adipose conversion in innumerable studies performed mainly in serum-containing media. Furthermore, the potentiating effect that occurs between glucocorticoids and arachidonic acid also accounts a posteriori for the high adipogenic activity exhibited with 3T3 (51) as well as Ob17 preadipocytes (11) by an ethanolic extract prepared from the serum of various species and which is now known to be enriched with both steroid hormones (51), fatty acids, and prostaglandins (14).

The adipogenic effect of glucocorticoids is not restricted to cells of preadipocyte clonal lines; it was also observed in primary cultures of precursor cells isolated from adipose tissue of various species including mouse (this study), rat (19), rabbit (23), and humans (21, 22). Moreover, when human cells were cultured in 5F medium supplemented with cortisol, an amplifying effect of arachidonic acid (5×10^{-6} M) led both to a two- to threefold increase in the GPDH activity level and the number of triacylglycerol-containing cells (D. Gaillard and R. Négrel, unpublished results). Glucocorticoids have also recently been shown to stimulate the differentiation of the multipotent clonal fetal rat cell line RCJ 3.1 into four distinct phenotypes of mesenchymal origin including bone, cartilage, muscle, and adipose tissue (52). Interestingly, whereas the muscle and bone phenotypes were expressed at a low frequency in the absence of dexamethasone, the formation of chondrocytes and adipocytes was absolutely dependent upon the addition of the glucocorticoid and showed a similar dose- and time-dependence as compared with that observed in our study.

The effect of glucocorticoids on cell differentiation is not restricted to adipose cells and is also known to control myogenesis in vitro through the stimulation of multiplication and/or differentiation of satellite progenitor cells (53–55). This stimulation appears to be related to some factor(s) released into the culture medium and acting according to an autocrine/paracrine mode (53). Differentiation of these satellite cells can take place in serum-free medium enriched with fetuin, a polyunsaturated fatty

acid (linoleic acid), and either hydrocortisone or dexamethasone (55). Since the involvement of some prostanoids and cyclic AMP in the control of proliferation and differentiation of myoblasts has been invoked (56), it is tempting to speculate that prostacyclin might also play a critical role in this differentiation process.

The inhibitory effect of a long-term treatment with high concentrations of glucocorticoids, as observed with RCJ 3.1 (52) as well as Ob1771 cells, has already been reported for 3T3-F442A preadipocytes (35, 57). Although the molecular basis of this inhibitory effect remains to be established, it is reasonable to propose that it might result from a direct negative control of these hormones at the level of gene expression; the involvement of the anti-inflammatory properties of glucocorticoids (see below) is unlikely since the inhibitory effect was reversed neither by arachidonic acid nor carbaprostacyclin (Table 2).

The promoting effect of corticosterone upon arachidonic acid metabolism in Ob1771 cells, which leads to an increase in the level of unmetabolized arachidonic acid and prostanoids including prostacyclin, could be surprising in the light of the well-known antiinflammatory properties of glucocorticoids. These properties are known to be linked to a diminution of arachidonic acid metabolism related to the ability of glucocorticoids to reduce the mobilization of the fatty acid through the expression of proteins of the lipocortin or calpactin family (58, 59). In fact, numerous works have challenged this view: first, the physiological significance of the phospholipase inhibition by calpactins has been severely questioned (60–62) and second, glucocorticoids fail to act according to this rule not only in vitro but also in vivo since these hormones have been indeed reported *i*) to be without effect upon prostaglandin synthesis (63, 64), *ii*) to stimulate arachidonic acid mobilization (65, 66), and *iii*) to increase prostaglandin synthesis and cyclooxygenase activity (67–72).

The rise in 6-keto-$PGF_{1\alpha}$ released from corticosterone-treated Ob1771 cells is likely to be related to an increase in the amount or activity of prostacyclin synthetase. This effect might be somewhat specific since general protein synthesis remained unaffected under these conditions (not shown) and PGE_2 production was not affected. Furthermore, it is interesting to note that unmetabolized labeled arachidonic acid and other metabolites as well as unknown hydrosoluble oxidation products also accumulated upon exposure of Ob1771 cells to corticosterone. This phenomenon, which might be due to a blockade of re-esterification reactions (see above and ref. 66), should contribute first to the enhanced availability of precursor arachidonic acid and second to a potentiation of the mitogenic-adipogenic effect of prostacyclin, since polyunsaturated fatty acids have been reported to behave as protein kinase C activators (73–75).

Altogether these results emphasize the role played by glucocorticoids in the control of terminal differentiation of adipose cells in vitro, as modulators of the autocrine action of prostacyclin, in agreement with the model that we have proposed (15) and recently validated (76). Glucocorticoids, which are known to affect the mass of various adipose depots in mammals (16, 17), should thus act in vivo on developing preadipocytes before any metabolic action on mature adipocytes (77, 78). It is proposed that these hormones are involved not only in the hypertrophic but also in the hyperplastic growth of adipose tissue. Such an involvement could explain the excessive development of intra-abdominal adipose tissue associated with hypercortisolism in Cushing's Syndrome (17, 78).

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