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METABOLISM OF ARACHIDONIC ACID AND PROSTAGLANDIN SYNTHESIS IN THE PREADIPOCYTE CLONAL LINE OB_{17}

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SUMMARY : Biosynthesis of prostaglandins in ob_{17} preadipose cells was studied in culture. Dihomo-γ-linolenic acid is exclusively converted to PGE_1 . Arachidonic acid behaves quantitatively as a more potent precursor, leading to the synthesis of PGE_2 and 6-keto-$PGF_{1\alpha}$ (stable product of prostacyclin). In all cases prostaglandin synthesis was confirmed directly by radioimmunoassay. This synthesis is maximal during the growth phase and decreases dramatically after confluence at a time where adipose conversion occurs, suggesting a possible relationship between both events.

INTRODUCTION

Prostaglandins inhibit hormone-induced lipolysis and cAMP* production in adipose tissue (1,2). In this tissue treated with lipolytic hormones a prostaglandin-like material is released (3). In the past, most of the work on prostaglandin synthesis in adipose tissue has been focused on the nature of the products formed under basal or stimulatory conditions (3-7). To our knowledge no study has been performed on the synthesis of PG during adipose tissue development *in vivo* or during adipose conversion of preadipocyte cell lines *in vitro*. Two years ago we reported the establishment of the preadipocyte clonal line ob_{17} isolated from epididymal adipocytes of genetically obese mice (8). These cells can be converted to adipose cells with morphological and biochemi-

*Abbreviations : cAMP, adenosine-3',5'-monophophate ; PG(s), prostaglandin(s), TxB_2 , thromboxane B_2 ; C20:4, arachidonic acid ; C20:3, dihomo-γ-linolenic acid (8,11,14-eicosatrienoic acid).

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cal characteristics of mature adipocytes, including self-modulation of insulin receptors (9,10) and dependence upon exogénous triacylglycerol as a preferential source of fatty acids (8,11). Accordingly ob_{17} cells offer an opportunity i) to study PG synthesis in preadipose and mature cells and ii) to establish if a relationship, if any, exists between PG synthesis and adipose conversion.

MATERIALS AND METHODS

Cell culture : Methods of cell growth, cell numbering and determination of cell protein content were as previously described (8). Where indicated, insulin (17 nM or 170 nM) was added to the standard medium (changed every other day unless otherwise stated) at confluence and thereafter in order to increase the rate and the extent of adipose conversion (8).

Extraction of prostaglandins : Culture medium and washes of the cells were pooled either after 24 h of labeling with radioactive precursors of PG (method I) or 24 h after removal of the labeling medium and replacement with fresh unlabeled medium (method II). In all cases pH was brought to 3.0 with 70% citric acid, lipids were extracted twice with 3 volumes of cyclohexane: ethylacetate (1:1), and organic phase dried under nitrogen.

Prostaglandin analysis and determination : In some experiments the residue was dissolved in ethanol, directly applied to silicagel plates and then developed to a height of 19 cm in the organic phase of ethylacetate/isooctane/acetic acid/water (10:5:2:10), with unlabeled or tritiated PG standards run in parallel. After exposure to iodine vapor, scraped areas were collected in 1 ml of methanol and counted with Picofluor (Packard Instruments S.A.). In most separations by TLC, PG fractions were first separated by silicic acid column chromatography (12). This procedure was also applied to unlabeled lipid extracts in order either to purify the total PG fraction or to separate PGE from PGF fraction (12) ; tritiated PGs were included as internal standards. RIA for PGE_2 and $PGF_{2\alpha}$ were performed on aliquots of suitable dilutions according to Russo-Marie et al (13). The characteristics of the antisera used have been previously reported (14). RIA for 6-keto-$PGF_{1\alpha}$ and TxB_2 were carried out on the total PG fraction using kits purchased from New-England Nuclear.

Extraction and analysis of cellular lipids : After removal of the medium used in labeling experiments, washed cells were collected in 0.1 N HCl and extracted according to Bligh and Dyer (15). The residues were applied to silicagel plates and developed in hexane : diethylether : formic acid (80:20:1) with lipid standards run in parallel ; scraped areas were counted as described above.

Materials : [^{3}H] arachidonic acid (112 Ci/mmol), [^{3}H] eicosa-8,11,14-trienoic acid (dihomo-γ-linolenic acid ; 120 Ci/mmol), [^{3}H] PGE_2 and [^{3}H]$PGF_{2\alpha}$ (160 Ci/mmol) were purchased from the Radiochemical Centre, Amersham. Unlabeled PG were kindly provided by Dr. J. Pike of the Upjohn Co. PGE_2 and $PGF_{2\alpha}$ antisera were from Institut Pasteur Production. Other compounds were

obtained as follows : culture medium and fetal calf serum from Gibco ; bovine insulin, indomethacin and dexamethasone from Sigma Chemical Co. ; silicic acid from Mallinkrodt ; organic solvents and silicagel 60 from Merck ; lipid standards from Nu Check Prep.

RESULTS

The capacity of ob_{17} preadipose cells to synthetize prostaglandins was first studied by using labeled arachidonic acid (C20:4) and dihomo-γ-linolenic acid (C20:3) as precursors (Table 1). Cells were maintained under various culture conditions allowing different stages of adipose conversion. Although C20:3 was incorporated to a higher extent into cellular lipids of growing cells as compared to C20:4 (x5 fold), it was less efficiently converted to PG (:4 fold). Control experiments showed that both PG and lipoxygenase products were found exclusively as released material in the culture medium.

The total incorporation of each precursor into lipids decreased after the cells reached confluence ; it is more apparent with C20:3 than with C20:4 (20 fold versus 7 fold). The total incorporation of C20:3 and C20:4 became similar when compared on a cell (or on a protein) basis, but again C20:3 was less efficiently converted to PG. The conversion of C20:4 to PG decreased with time in culture (Table 1, last column, experiments 5 to 7), whether or not insulin was included in the medium.

In contrast C20:4 incorporation into cellular lipids increased with time in culture (experiments 3 and 6) and was enhanced significantly at day 3 and day 12 in the presence of insulin (experiments 5 and 7).

Polar lipids were the major radioactive products containing labelled C20:3 or C20:4 under all conditions. Among cellular lipids, a shift of incorporation from triacylglycerol to cholesterol esters, diacylglycerol and unesterified fatty acids was

Table I: Incorporation of labeled precursors into cellular lipids and released prostaglandins

All experiments were performed in 60 mm culture dishes except in experiment 6 (100 mm dishes). Cell number and cell protein content were respectively : experiments 1 and 2 : 10^5 and 0.145 mg ; 3 and 4 : 4 x 10^5 and 0.58 mg ; 5 : 5 x 10^5 and 0.145 mg ; 3 and 4 : 4 x 10^5 and 0.58 mg ; 5 : 5 x 10^5 and 0.6 mg ; 6 : 1.3 x 10^6 and 2 mg ; 7 : 9 X 10^5 and 0.99 mg. The total radioactivity present at the start of each experiment (total added) ranged from 4.5 µCi to 5.9 µCi, except in experiment 4 (7.5 µCi). 24 h later culture mediurm and washes were pooled, extracted and analyzed for PGs. Cells were collected separately, lipids extracted and separated (Materials and Methods). Numbers are the mean of duplicate dishes ; their values did not differ by more than 5%.

			RADIOACTIVITY INCORPORATED INTO :									
					Cellular lipids					Released prostaglandins		
Culture Conditions	Experiment number	Labeled precursor	Total incorporation		Polar lipids	Diacyl-glycerol	Unesterified fatty acid	Triacyl-glycerol	Cholesterol esters	Total		
			d.p.m.x10^{-3} (% of total added)	d.p.m./ 10^3cells	% of total incorporated					d.p.m.x10^{-3}	d.p.m./ 10^3cells	
Exponential phase (standard medium)	1.	C20:4	1035 (9.7)	10350	71.3	2.1	1.8	19.7	1.4	322	3220	
	2.	C20:3	4675 (47.9)	46750	79.8	1.4	1.3	14.3	1.2	77	770	
2 days post-confluence (standard medium)	3.	C20:4	605 (5.7)	1510	68.5	9.0	8.7	1.6	8.0	518	1295	
	4.	C20:3	930 (5.6)	2325	74.5	4.8	10.8	1.4	6.2	258	645	
3 days post-confluence (standard medium + 17 nM insulin)	5.	C20:4	8740 (66.7)	17480	76.5	1.0	10.5	10.7	0.3	127	254	
7 days post-confluence (standard medium)	6.	C20:4	5270 (42.8)	4050	71.6	2.0	15.5	10.3	0.2	418	320	
12 days post-confluence (standard medium + 170 nM insulin)	7.	C20:4	8050 (61.5)	8940	60.6	1.3	13.0	24.0	0.1	176	195	

first observed between growing cells and early confluent cells. Subsequently, during adipose conversion and net triacylglycerol accumulation (8), a shift of incorporation of C20:4 toward triacylglycerol was clearly visible at the expense of polar lipids (experiments 5 to 7).

TLC analyses (Fig. 1) were performed on culture media from exponentially growing ob_{17} cells, after labeling of the cells with C20:4 (A) or C20:3 (B). With C20:4 the two major radioactive PGs comigrated with standard PGE_2 and 6-keto-$PGF_{1\alpha}$, the end product of PGI_2. Under the same conditions C20:3 was only converted to a product which comigrated with PGE_1. Identical patterns of C20:4 conversion were obtained on early confluent cells (Fig. 2) and late differentiated cells (not shown). The ratio of radioactivity recovered into PGE_2 versus the 6-keto derivative ranged from 2 to 3 in all experiments, suggesting that

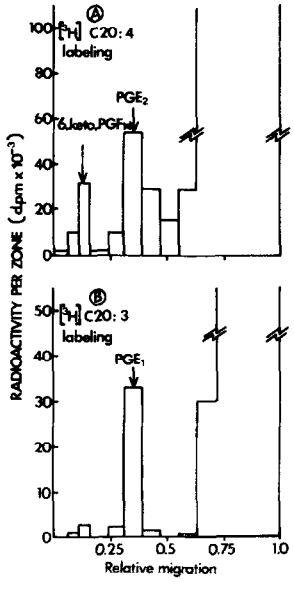

Fig. 1 : Identification of prostaglandins by TLC

Cell supernatants (culture medium + washes) from experiments 1(A) and 2(B) (see Table I) were extracted and directly analyzed by TLC as described in "Materials andMethods". The zone with an Rf from 0.6 to 1.0 corresponds to unesterified fatty acids, neutral lipids and unidentified lipoxygenase products.

no quantitative changes in the PGs formed occurred during the course of differentiation.

In addition to comigration with authentic PG standards, the radioactive products were identified by the following criteria i) the amounts of radioactive material were reduced markedly or even became insignificant when cells were prelabeled with C20:4 and then maintained for 24 h in the presence of 0.25 µM dexamethasone or of 12.5 µM indomethacin (Figs. 2C and 2B) ii) the radioactivity comigrating with PGE_2 was quantitatively converted to a new product comigrating with $PG(A_2)B_2$ upon potassium hydroxyde treatment and with $PGF_{2\alpha}$ after reduction by sodium borohydride (not shown) iii) in the absence of labeled precursor, PGE_2 and 6-keto-$PGF_{1\alpha}$ were identified by radioimmunoassay in the culture medium (Table 2).

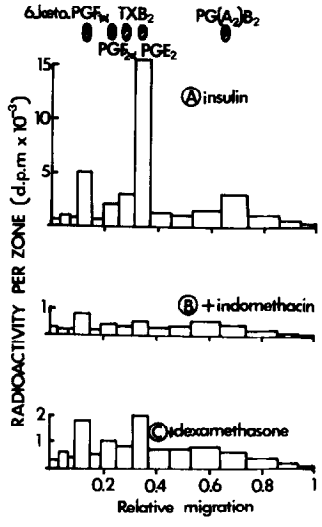

Fig. 2 : Prostaglandin production by 3 day post-confluent ob17 cells prelabeled with [^{3}H] arachidonic acid

After 24 h in the presence of [^{3}H]arachidonic acid (5.9 µCi in 60 mm dishes containing 4 ml of standard medium plus 17 nM insulin), 5 x 10^5 cells were washed and fed with fresh standard medium containing either 17 nM insulin alone (A), or 17 nM insulin supplemented with 12.5 µM indomethacin (B) or with 0.25 µM dexamethasone (C). 24 h later, cell supernatants were extracted, total PG fraction eluted by silicic acid column chromatography and subsequently analyzed by TLC (see "Materials and Methods"). Note the differences in scale on the ordinates.

TABLE II: Radioimmunoanalysis of released prostaglandins from 2 day post-confluent ob17 cells

Addition to standard medium plus 17 nM insulin	PGE_2	6-keto-$PGF_{1\alpha}$	$PGF_{2\alpha}$	TxB_2
	ng/mg protein/24 h			
None	80 ± 8.0	28 ± 3.0	4.0 ± 1.0	N.D.
12.5 μM indomethacin	2 ± 0.5	3 ± 1.0	N.D.	N.D.
0.25 μM dexamethasone	3 ± 1.0	5 ± 1.5	N.D.	N.D.

Cell media, removed 24 h after feeding the cells as indicated, were processed as described under "Materials and Methods" for RIA. 60 mm culture dishes contained about 450 000 cells (0.7 mg protein) in 4 ml of each medium. Values are the mean of four separate dishes under each condition ± S.E.M. 50% displacement were obtained with 7, 130, 25 and 50 pg for PGE_2, 6-keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$ and TxB_2 respectively. N.D. : not detectable.

The ratio of net PGE_2 formed versus 6-keto-$PGF_{1\alpha}$ (2.8) was in agreement with that previously observed by the use of radioactive precursors (Fig. 1A and Fig. 2A). TxB_2 was undetectable and $PGF_{2\alpha}$ was present in small amounts. It can be assumed that PGE_2 levels determined by radioimmunoassay do indeed represent immunoreactive PGE_2 since crossreactivity of PGE_1 with PGE_2 antiserum was only 3.2%. It is unlikely that significant PGE_1 levels were formed under these conditions, given the low conversion of C20:3 into PGE_1 (Table 1). As expected, indomethacin and dexamethasone were able to reduce considerably the net production of PGE_2 and to a lesser extent that of 6-keto-$PGF_{1\alpha}$.

Time course production of PGE_2 - the major PG synthetized - was quantitated by radioimmunoassay throughout the period of exponential growth (Fig. 3). Total PGE_2 biosynthesis increased with time during the 24 h period and reached a value of 400 ng PGE_2/dish corresponding to 1 μg/mg protein or 1.45 μg/10^6 cells. The rate of PGE_2 production was calculated per hour and normalized according to protein content (or to cell number) at each time. Fig. 3A shows a decreasing rate of production with increasing cell density.

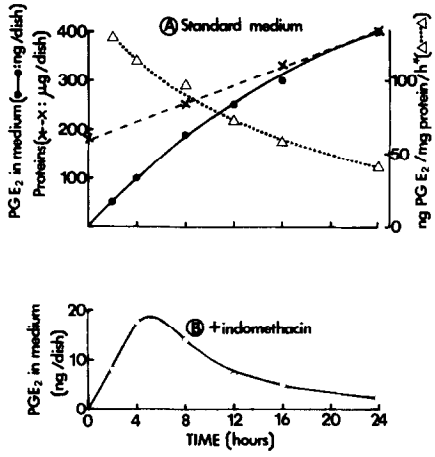

Fig. 3 : PGE_2 biosynthesis by exponentially growing ob_{17} cells

1.25×10^5 cells in 100 mm culture dishes were fed with 10 ml of fresh standard medium (time zero of the curves). 1 ml aliquots were withdrawn at indicated times and processed separately for RIA of PGE_2 . When present, 12.5 µM indomethacin (B) was added at the start of the experiment. Cell number (2.75×10^5 cells at the end of the experiment) and protein content were determined in parallel on separate dishes. Each point is the mean of two determinations on triplicate dishes ; their values did not differ by more than 10%. * assuming a linear production with time, released PGE_2 was calculated per mg protein per hour.

This decrease was dramatically pronounced (about 10 fold decrease) when cells reached the resting state (Tables 1 and 2). The level of PGE_2 was reduced by 75% during the first 4 hours of culture in the presence of indomethacin and decreased to an insignificant level after 24 h, although growth was essentially unaffected (Fig. 3B).

DISCUSSION

C20:4 is a better precursor of prostaglandins than C20:3 in preadipose ob_{17} cells. In adipose converted cells, incorporation of C20:4 occurs predominantly into polar lipids, in contrast to palmitate and oleate which are mainly incorporated into neutral lipids (8,11). The incorporation of C20:4 into polar lipids indicates a preferential mobilization from these compounds for PG biosynthesis, as already known for other tissues (16). However the enhanced incorporation of C20:4 into triacyl-

glycerol, observed when differentiating cells are maintained in the presence of insulin, suggests that some mobilization of arachidonyl acyl group could also occur from neutral lipids, as previously suggested (4,17).

The finding that PGE_2 is the main prostaglandin produced in ob_{17} cells is in agreement with results obtained on rat (3-7) and rabbit (18) adipose tissue. Moreover low levels of $PGF_{2\alpha}$ and an undetectable level of TxB_2 are in agreement with most studies, although some discrepancies have been reported for $PGF_{2\alpha}$ which was shown to attain significant levels (5-7,19). The production of 6-keto-$PGF_{1\alpha}$, likely to be present as unidentified material released from rat adipose tissue (6), was briefly reported in isolated rat adipocytes (20) and in 3T3-L1 mouse preadipocytes (21) during the course of this study.

The rate of PGE_2 and 6-keto-$PGF_{1\alpha}$ synthesis is inversely related to cell density (Fig. 3) and becomes very low after confluence (Tables 1 and 2). This observation is in agreement with previous studies on rabbit renomedullary interstitial cells (22) and on mouse Balb/C 3T3 normal fibroblasts (23). Moreover, indomethacin blocks PG production in ob_{17} cells without affecting cell growth as previously reported with other cultured cell types (22,24,25). Therefore PG production seems not to be a prerequisite for growth but rather a consequence of cell proliferation. However, it cannot be excluded that the decreasing capacity of ob_{17} cells to synthetize prostaglandins in the resting state could be in some way related to their committment to enter their differentiation program. Investigations are currently being performed to test this hypothesis.

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