Arachidonic Acid Metabolites of the Lipoxygenase as Well as the Cyclooxygenase Pathway May Be Involved in Regulating Preadipocyte Differentiation

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Conditions that trigger preadipocyte differentiation in vivo have yet to be elucidated. To investigate the role of endogenous arachidonic acid (AA) metabolites on adipose tissue growth, rat preadipocytes in primary culture were induced to differentiate using medium conditioned by isolated mature adipocytes (ACM). Differentiation was determined by assay of glycerol-3-phosphate dehydrogenase (GPDH). When collected in the presence of indomethacin (10 nmol/L) to inhibit prostaglandin (PG) synthesis by adipocytes, ACM induced greater differentiation (GPDH activity, 405 ± 68 nmol NADH used/min/mg protein) than when indomethacin was added postcollection to inhibit preadipocyte PG synthesis (205 ± 24, $P < .05$) or ACM alone (304 ± 55). This suggested that PGs released by adipocytes inhibited differentiation, whereas those released by preadipocytes appeared to act in an autocrine manner to stimulate differentiation. However, 24-hour collections of ACM contained 125 pmol/L PGE_2 and 900 pmol/L PGI_2, concentrations too low to promote differentiation when added exogenously. Nordihydroguaiaretic acid (NDGA; 10 μmol/L), an inhibitor of lipoxygenase (LOX), stimulated the ACM-induced increase in GPDH activity (ACM, 99 ± 13; ACM + NDGA, 369 ± 130). In contrast, when differentiation was induced by a hormonal cocktail (MIX), including insulin and corticosterone, NDGA decreased GPDH activity (MIX, 329 ± 66; MIX + NDGA, 142 ± 40; $P < .03$). We concluded that preadipocyte differentiation within adipose tissue may be subject to both positive and negative regulators derived from AA metabolism resulting from both LOX and cyclooxygenase (COX) activity.

ALTHOUGH MUCH IS KNOWN about preadipocyte differentiation in vitro, the conditions that trigger preadipocyte differentiation in vivo have yet to be elucidated, but may include locally released factors that act in a paracrine or autocrine manner. We have previously demonstrated that preadipocyte replication is stimulated by factors released from microvascular endothelial cells,[1] while differentiation is induced by extracellular matrix secreted by the endothelial cells,[2] as well as in response to factors released by mature adipocytes.[3,4] Thus, the microenvironment within adipose tissue most likely contains numerous bioactive components, the balance between which at any one time determines whether differentiation will proceed.

A possible contribution to the bioactivity of this microenvironment may be from prostaglandins (PGs), which are known to be synthesized and secreted by preadipocytes and adipocytes.[5,6] These arachidonic acid (AA) metabolites have been shown to modulate preadipocyte differentiation. Exogenous prostacyclin (PGI_2) induced the differentiation of Ob1770 cells[7] and, more recently, rat preadipocytes in primary culture.[5] However, the effective concentration of PGI_2 was 100-fold greater than the amount measured in adipose tissue by microdialysis.[6] In contrast, PGE_2 and $PGF_{2\alpha}$ inhibited the differentiation of the adipogenic cell line 1246 and rat preadipocytes.[8] The latter observation is consistent with earlier reports that indomethacin, an inhibitor of PG synthesis, triggered the differentiation of 3T3-L1,[9] ob 17,[10] and TA1 cells.[11] Another prostaglandin, PGJ_2, has also recently been shown to stimulate differentiation.[12,13] PGs may, therefore, act by autocrine or paracrine mechanisms to modulate adipogenesis.

PGs are synthesized from AA via the cyclooxygenase (COX) pathway, which can be blocked by indomethacin. Another metabolic fate for AA is via the lipoxygenase (LOX) pathway, which results in the production of leukotrienes and hydroxyeicosatetraenoic acids (HETEs). A recent study has shown that 8(s)-HETE strongly activates the peroxisome proliferator-activated receptors (PPAR).[14] $PPAR_{\gamma 2}$ is expressed very early in preadipocyte differentiation and plays an important role in mediating transcriptional activation of differentiation-dependent genes.[15] 8(s)-HETE was also shown to induce the differentiation of 3T3-L1 preadipocytes.[14] To our knowledge, the only other study on the effects of LOX metabolites and inhibitors on preadipocyte differentiation reported that they were without effect on 1246 cells.[16]

Although it is known that exogenous PGs and 8(s)-HETE have the ability to influence preadipocyte differentiation, it is not clear whether they play a role in the regulation of adipose tissue growth in vivo. Therefore, to reproduce as closely as possible the endogenous eicosanoid components and concentrations of the adipose tissue microenvironment, we have induced preadipocyte differentiation using a medium conditioned by adipocytes (ACM).[3] The aim of the present study was to determine if PGs produced by mature adipocytes act to regulate preadipocyte differentiation. In addition, we have examined the effect of LOX inhibition to determine whether endogenous products of the LOX pathway are active in the regulation of preadipocyte differentiation.

METHODS

All experiments were performed using rat preadipocytes in primary culture. Male Sprague-Dawley rats (Charles River Breeding Laboratories Canada, Montreal, Canada) (body weight, ~300 g) were fed standard rat chow ad libitum and maintained on a 12:12-hour light-dark cycle at 22°C. The rats were killed by cervical dislocation under halothane anesthesia, and epididymal and retroperitoneal fat pads were

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resected using sterile procedures. The fat pads from each animal were pooled for isolation of adipocytes or preadipocytes.

Preadipocyte Culture

Stromal vascular cells were isolated from the fat pads and cultured as previously reported.[4] Briefly, adipose tissue was finely minced and digested with 1 mg/mL type II collagenase (Sigma Chemical, St Louis, MO) in Hank's balanced salts solution (HBSS; Gibco, Burlington, Canada) supplemented with penicillin (63 mg/L) and streptomycin (143 mg/L), with gentle shaking at 37°C for 45 minutes. The cell suspension was filtered through a 250-μm Nitex mesh (B & SH Thompson, Scarborough, ON), centrifuged at 200 *g* for 10 minutes, the resuspended cell pellet filtered through a 25-μm mesh, then recentrifuged. The pellet obtained consisted mainly of preadipocytes, which were enumerated using an electronic counter (Coulter Electronics, Hialeah, FL) and seeded in 24-well plates at an approximate density of 4×10^4 cells/cm^2 in 1 mL/well α-Minimum Essential Medium (αMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) and antibiotics as described earlier. Cells were propagated at 37°C in a humidified atmosphere of 95% air, 5% CO_2. The medium was replaced every other day.

ACM Preparation

Rat retroperitoneal and epididymal fat pads were resected, pooled, and digested for 1 hour at 37°C with collagenase (1 mg/mL, type II) in Krebs-Ringer HEPES buffer (pH 7.4) containing 2 mmol/L glucose, 200 nmol/L adenosine, and 1% bovine serum albumin (BSA) as previously described.[3] The digest was filtered through a 150-μm mesh and the floating adipocytes washed thrice and suspended (vol/vol) in one-quarter volume αMEM supplemented with 10% FBS and 4% BSA (ie, 4 mL packed cells + 1 mL medium). Culture flasks were pre-wetted with the same medium and approximately 4×10^5 adipocytes were inoculated per 25 cm^2. Flasks were quickly inverted to allow adherence of cells to the flask. After about 90 minutes at 37°C, the flasks were turned over and fresh αMEM plus 10% FBS (5 mL/25 cm^2) gently added with or without indomethacin. The medium conditioned by the adipocytes was collected 48 hours later.

Initially, 100 μmol/L indomethacin was used, as this concentration was reported to promote preadipocyte differentiation.[11,16] After it became apparent that this high concentration of the drug was affecting differentiation independent of its inhibition of PG synthesis, the concentration was reduced to 10 nmol/L. We found that this concentration was sufficient to block PG synthesis by greater than 80%, yet have no direct effect on differentiation.

Preadipocyte Differentiation

Differentiation was initiated on day 4 after seeding, when the preadipocytes were confluent. Cells were exposed to fresh αMEM plus 10% FBS (control), to ACM (1 mL/well) with or without indomethacin (10 nmol/L or 100 μmol/L), or to a standard differentiation medium (MIX) that consisted of αMEM plus 10% FBS supplemented with insulin (10 nmol/L), corticosterone (100 nmol/L), and isobutylmethylxanthine (.5 mmol/L) for 2 days followed by a medium containing .5% Liposyn, a lipid emulsion (Abbott Laboratories, Montreal, Canada), in addition to insulin and corticosterone.

To distinguish between the effects of PGs produced by mature adipocytes in ACM and those produced by the differentiating preadipocytes, indomethacin (10 nmol/L or 100 μmol/L) was added directly to the preadipocytes in some cases.

Inhibition of LOX

Nordihydroguaiaretic acid (NDGA; 10 μmol/L), an inhibitor of LOX,[17,18] was added to preadipocytes at the initiation of differentiation using either MIX or ACM in the presence or absence of indomethacin (100 μmol/L), and each time media were changed.

Assessment of Preadipocyte Differentiation

Cells were harvested on day 10 after seeding for assessment of differentiation by assay of glycerol-3-phosphate dehydrogenase (GPDH) activity (EC 1.1.1.8). Cells were homogenized in 10 mmol/L HEPES buffer containing .25 mol/L sucrose, 1 mmol/L EDTA, and 1 mmol/L dithiothreitol, and centrifuged at 100,000 × *g* at 0 to 4°C for 20 minutes. The floating fat layer was discarded. Aliquots of the supernatant were assayed for GPDH activity under optimal substrate and zero-order kinetic conditions by the method of Kozak and Jensen.[19] One unit of enzyme activity corresponded to the oxidation of 1 nmol of NADH/min. Protein concentration of the supernatant was determined by the Coomassie blue method,[20] and the specific activity of each enzyme was expressed as units per milligram protein.

Identification and Assay of Endogenous PGs

To identify which PGs are synthesized and released by preadipocytes and adipocytes, cells were incubated in the presence of [1-^{14}C] AA for 30 minutes at 37°C. Following centrifugation to remove the cells, labeled PGs were extracted from the incubation medium by shaking twice with diethyl ether/methanol/.2 mol/L citric acid (30:4:1, vol/vol/vol). The organic layer was removed, evaporated to dryness under nitrogen, and the extracted lipids redissolved in 50 μL solvent (ethyl acetate/2.2.4-trimethyl pentane/acetic acid/water, 110:50:20:100 vol/vol/vol/vol. After the organic layer was saturated with water by shaking, the excess water was removed). The extracts were applied to silica gel thin-layer chromatography (TLC) plates, together with standard PGs, and the plates were run in the same solvent. Standard PGs were visualized in iodine vapor and the labeled samples by autoradiography.

PGs identified by TLC, namely, PGE_2, and PGI_2 (detected as 6-keto-$PGF_{1\alpha}$, a stable metabolite) were then quantitated in media conditioned by adipocytes and preadipocytes for 24 hours. PGs were extracted according to the manufacturer's instructions and measured using a radioimmunoassay kit (Amersham Canada, Oakville, Canada).

Determination of Effective Concentrations of PGs

To determine the concentrations of PGs required to stimulate or inhibit differentiation, confluent preadipocytes were exposed to exogenous PGs under serum-free conditions. The concentration range tested was 10^{-10} mol/L to 10^{-6} mol/L. A stable analog, carbacyclic PGI_2 ($cPGI_2$; Sigma Chemical), was used because of the short life of PGI_2. Preadipocytes were allowed to grow in a medium supplemented with 10% FBS until confluent 5 days after seeding. This medium was then replaced with serum-free Dulbecco's Modified Eagle Medium/Ham's F12 (DMEM-F12; Gibco), supplemented with transferrin (10 μg/mL), triiodothyronine (200 pmol/L), and insulin (500 nmol/L) (ITT).[2] PGs were added at the same time and replaced every 2 days when media were changed. Cells were harvested for GPDH assay on day 14 after seeding.

Statistical Analysis

Data were analyzed by analysis of variance and Tukey's method for multiple comparisons. All determinations were made in at least triplicate, and n values represent the number of trials. In every experiment, cells from the same batch were exposed to all the experimental conditions.

RESULTS

High-Concentration Indomethacin Augments Preadipocyte Differentiation

The addition of 100 μmol/L indomethacin to preadipocytes at confluence significantly increased both MIX- and ACM-induced differentiation (threefold to fourfold), as assessed by GPDH activity (Fig 1A). Differentiation was also increased by indomethacin in cells under control (10% FBS) conditions. Furthermore, there was a trend suggesting that GPDH activity induced by ACM collected in the presence of indomethacin was greater (~20%) than that induced by ACM when indomethacin was added after collection, ie, directly to the preadipocytes (Fig 1A).

Low-Concentration Indomethacin Inhibits Preadipocyte Differentiation

Differentiation induced by MIX was reduced by the addition of 10 nmol/L indomethacin, yet had no effect on control cells grown in a medium supplemented with 10% FBS alone

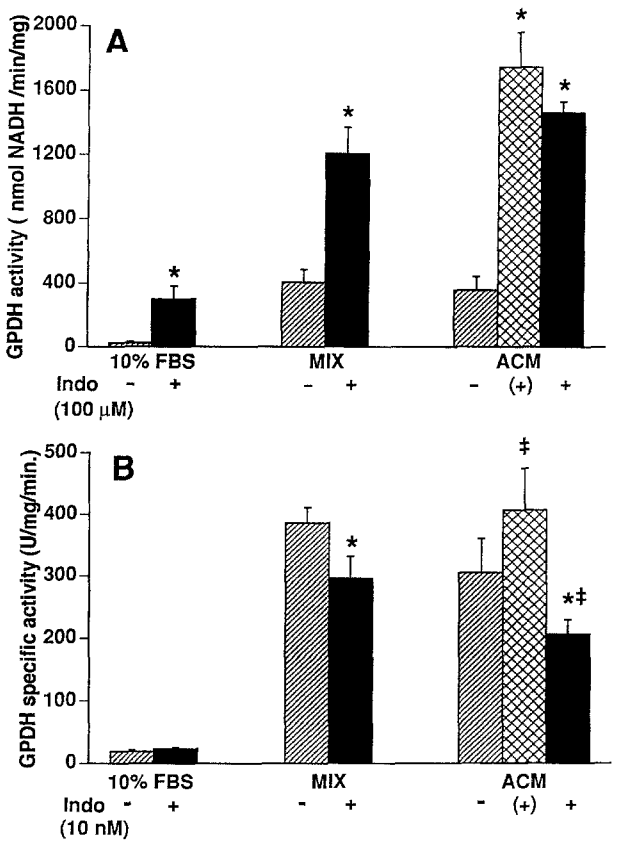

Fig 1. Effects of indomethacin on preadipocyte differentiation, expressed as GPDH specific activity (1 U activity = 1 nmol NADH used). Differentiation was induced by the addition of MIX including insulin and corticosterone (MIX) or by ACM in the absence (▨) or presence of indomethacin (Indo; ■) added directly to the preadipocytes to inhibit PG synthesis by these cells. An additional group (▩), indicated by (+) Indo, for which indomethacin was added during the collection of ACM to inhibit PG synthesis by adipocytes, is also shown. (A) Indomethacin: 100 μmol/L (B) indomethacin 10 nmol/L. *Values with indomethacin that are significantly different from those without indomethacin. ‡Significantly different, $P < .05$, n = 5.

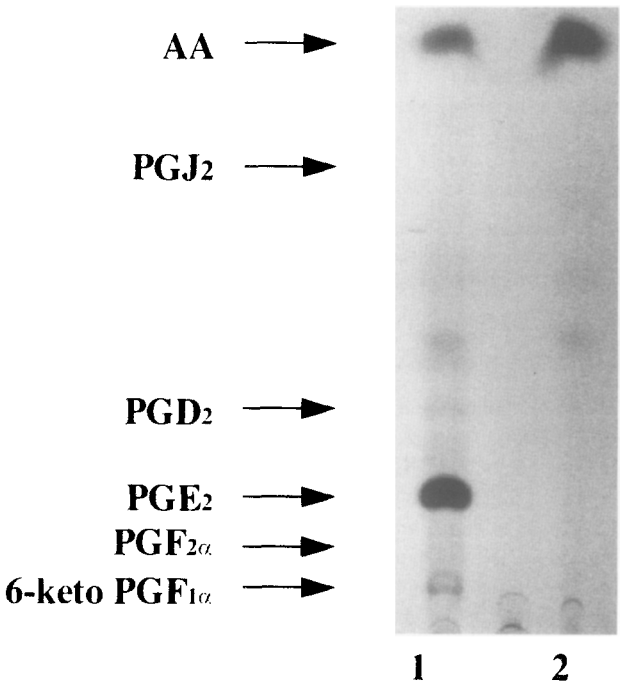

Fig 2. Arachidonate metabolites released by preadipocytes in culture. TLC of [1-^{14}C]-labeled PGs released by preadipocytes in the absence (Lane 1) or presence (Lane 2) of indomethacin (10 μmol/L) during a 30-minute period on day 4 of culture. Cells were incubated with [1-^{14}C] AA; the released PGs were then extracted and applied to silica gel TLC plates, together with standard PGs. The plates were run in ethyl acetate/2.2.4-trimethyl pentane/acetic acid/water (110:50:20: 100, vol/vol/vol/vol). The labeled samples were visualized by autoradiography and are compared with standard PGs, which were visualized in iodine vapor (arrows).

(Fig 1B). Similarly, this concentration of indomethacin significantly decreased ACM-induced differentiation when added directly to the preadipocytes. In contrast, addition of indomethacin to mature adipocytes during the collection of ACM significantly increased the differentiation induced compared with when indomethacin was added after collection (Fig 1B).

Detection of Released PGs

Metabolites of [1-^{14}C] AA released from preadipocytes in the absence or presence of indomethacin are shown in Fig 2. Bands seen in lane 1 that are absent in lane 2 may represent PGs. Thus, preadipocytes on day 4 after culture were found to release PGE_2 and PGI_2 (as 6-keto-$PGF_{1\alpha}$) into the medium. Trace amounts of PGD_2 and $PGF_{2\alpha}$ were also detected by TLC. Bands seen in lane 2 (Fig 2) in the presence of indomethacin may be LOX products. Release of PGs by mature adipocytes was undetectable, and this may have been due to incorporation of most of the [1-^{14}C]-AA into the triacylglycerol content of the cells, rather than into membrane phospholipids, but this also may have been due to concentrations of PGs below the detection limit of this method (see later).

PG Concentration in Conditioned Media

With the ratio of the number of cells to the volume of media the same as in cell-culture studies, the concentrations of PGE_2 and PGI_2 (measured as 6-keto-$PGF_{1\alpha}$) in a 24-hour collection of

ACM were 125 pmol/L and 900 pmol/L, respectively. The concentration of PGE_2 in media conditioned by confluent preadipocytes for 24 hours was approximately 11-fold greater than that in ACM, while that of PGI_2 was not different (PGE_2, 1,400 pmol/L; PGI_2, 1,300 pmol/L).

Effective Concentrations of Exogenous PGs

In a serum-free medium, PGE_2, PGI_2, and PGJ_2 stimulated differentiation only at the highest concentration tested (1 μmol/L (Fig 3). In contrast, $PGF_{2\alpha}$ inhibited preadipocyte differentiation at a concentration 100-fold lower (10 nmol/L).

Inhibition of LOX With NDGA

Differentiation induced by MIX was inhibited in the presence of 10 μmol/L NDGA (Fig 4). The addition of 100 μmol/L indomethacin to MIX-treated cells stimulated a threefold increase in GPDH activity, which was also inhibited by NDGA (Fig 5). In contrast, when cells were differentiated with ACM, addition of NDGA tended to increase GPDH activity (Fig 4). This difference did not reach statistical significance, due to the large degree of variability between batches of cells, although the greater than threefold increase strongly suggests a trend. Furthermore, NDGA failed to inhibit the increased differentiation induced by ACM in the presence of 100 μmol/L indomethacin (Fig 5).

DISCUSSION

Our results demonstrate that metabolites of AA, the eicosanoids, may exert both positive and negative effects within the microenvironment of adipose tissue to regulate preadipocyte differentiation. We have demonstrated that these metabolites are probably the products of the LOX, as well as the COX, pathways of AA metabolism.

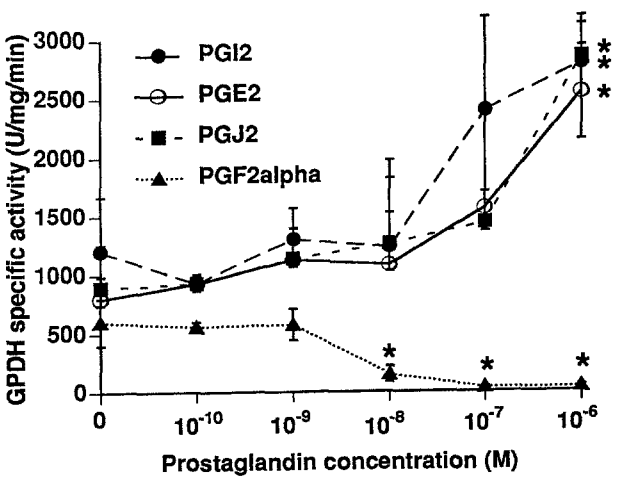

Fig 3. Effect of PGE_2, PGI_2, PGJ_2, and $PGF_{2\alpha}$ on rat preadipocyte differentiation in primary culture. Preadipocytes were cultured in medium supplemented with 10% FBS until confluent, then differentiated in a serum-free ITT medium and exposed to increasing concentrations of PG. Cells were harvested at day 14 after culture and assayed for GPDH activity. Each point represents the mean ± SE of at least 3 trials. *Values significantly different from zero control (no PG added), $P < .05$.

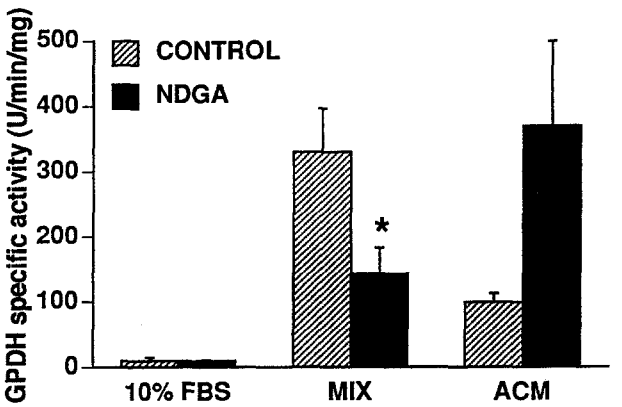

Fig 4. Effect of a LOX inhibitor, NDGA, on MIX- and ACM-induced preadipocyte differentiation. Preadipocytes were cultured in medium supplemented with 10% FBS until confluent then differentiated using MIX (n = 11) or ACM (n = 5) in the presence or absence of 10 μmol/L NDGA. Cells were harvested at day 10 after seeding and assayed for GPDH activity. *Value significantly different from the same condition in the absence of NDGA, $P < .03$.

By using ACM to differentiate preadipocytes, we have attempted to reproduce in vitro the endogenous PG components and concentrations of adipose tissue. The addition of indomethacin (100 μmol/L) to adipocytes during the collection of ACM, compared with addition after its collection, resulted in a greater induction of GPDH activity (Fig 1A). This trend suggested that the PGs secreted by adipocytes acted to inhibit differentiation. However, the threefold to fourfold increase in GPDH activity induced by MIX, together with the stimulation of differentiation in cells with 10% FBS alone (Fig 1A), implies that indomethacin-induced differentiation by a mechanism unrelated to PG synthesis; indeed, this has recently been reported.[21] Indomethacin has

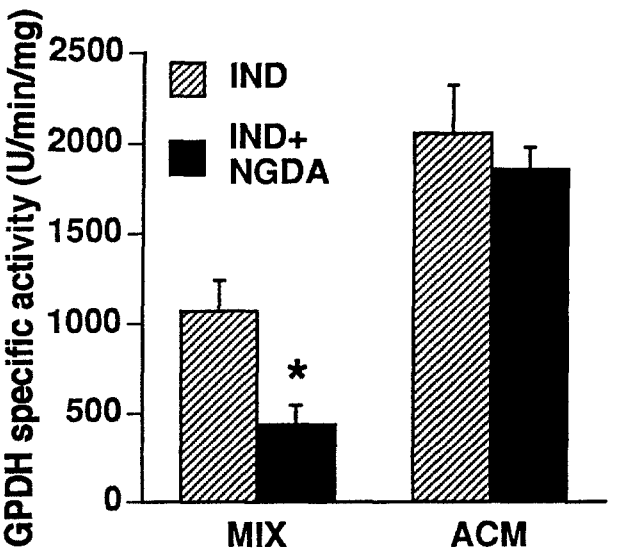

Fig 5. Effect of a LOX inhibitor, NDGA (10 μmol/L), on MIX- and ACM-induced preadipocyte differentiation in the presence of indomethacin (100 μmol/L). Cells were cultured as described in Fig 3 (n = 5). *Value significantly different from the same condition in the absence of NDGA, $P < .05$.

been found to bind and activate PPARγ. This highly adipogenic activity may have partly masked the effect of blocking inhibitory PG synthesis. Therefore, we repeated the study using a concentration of indomethacin, which we found would still inhibit PG synthesis, yet have no direct effect on differentiation.

When the low concentration of indomethacin (10 nmol/L) was used, it became apparent that adipocytes synthesize and release inhibitory PGs, whereas preadipocytes release stimulatory PGs (Fig 1B). Although PGI_2 has been shown to be secreted within adipose tissue in vivo and to stimulate differentiation in vitro,[5,6] our data suggest that adipocytes secrete insufficient amounts of this PG, or that its half-life is too short to contribute significantly to the positive activity of ACM.

The PGs, PGE_2 and $PGF_{2\alpha}$, have been reported to be negative regulators of differentiation.[8] However, in the present study, PGE_2 was found to stimulate differentiation, although only at very high, probably pharmacological, concentrations and under serum-free conditions (Fig 3). On the other hand, $PGF_{2\alpha}$ reduced differentiation at a concentration that may be attained locally within adipose tissue (10 nmol/L). Thus, it is possible that the inhibitory prostanoid activity released by adipocytes may be due to $PGF_{2\alpha}$, although this PG was not detected by TLC. Preadipocytes were found to synthesize a significantly greater amount of PGE_2 than adipocytes, but it is not clear whether this amount was sufficient to induce the positive effect on differentiation suggested by these data. The concentration of the various PGs in the interstitial spaces of adipose tissue in vivo is the additive product of secretion by the three major cell types of the tissue and might be expected to be somewhat higher than those measured in in vitro studies. Nevertheless, data obtained from in situ microdialysis of rat adipose tissue suggests that under basal or stimulated (by angiotensin II) conditions, the concentrations of PGI_2 and PGE_2 may still be too low to significantly affect preadipocyte differentiation.[6] On the other hand, elevated secretion of either inhibitory or stimulatory PGs may occur under certain conditions that have not yet been identified.

The overall effect of ACM on differentiation is positive, despite the apparent presence of an inhibitory PG component. This positive effect was augmented by indomethacin, the action of which, in addition to blocking the synthesis of inhibitory PGs, may be due to the subsequent redirection of AA as substrate through an alternative metabolic route such as the LOX pathway. To test this hypothesis, we performed similar experiments in the presence of the LOX inhibitor, NDGA. These studies provided some exciting, novel results. NDGA had opposite effects on MIX- and ACM-induced differentiation (Fig 4), while having no effect by itself. With or without indomethacin, NDGA reduced GPDH activity induced by MIX, suggesting that stimulatory leukotrienes, HETEs, or other products of LOXs are formed in response to MIX (Fig 5). Furthermore, the addition of indomethacin appeared to augment production of these metabolites, thus supporting our hypothesis. Serrero et al reported that pharmacological concentrations of leukotrienes B4 and C4 had no effect on the differentiation of 1246 preadipocytes,[16] whereas 8(s)-HETE has been shown to activate $PPAR_{\gamma 2}$ and to induce 3T3-L1 preadipocyte differentiation.[14] Thus, it seems more likely that the active metabolites may be HETEs, but whether these are formed in adipose cells is unknown. It is also possible that NDGA induced the observed changes by a mechanism unrelated to LOX inhibition. Other actions of NDGA, such as inhibition of platelet-derived growth factor–stimulated DNA synthesis and tyrosine phosphorylation have been reported.[22]

In contrast to the effect with MIX, when cells were differentiated using ACM, NDGA had the opposite effect and tended to increase GPDH activity by more than threefold. Moreover, NDGA failed to inhibit the increased differentiation induced by indomethacin. These data imply that ACM induced differentiation by a different mechanism than MIX, an observation we have made previously.[4] However, these results are more difficult to interpret. On the one hand, it would appear that ACM induced inhibitory LOX metabolites that were blocked by the NDGA, thus promoting differentiation (Fig 4). On the other hand, the lack of an NDGA effect on the indomethacin-stimulated increase of ACM-induced differentiation (Fig 5) implies that, in this case, indomethacin promoted differentiation by a mechanism that did not involve LOX metabolites. It is possible that indomethacin produced an effect of such magnitude (20-fold increase) that the concentration of NDGA was inadequate to block it. Whatever the interpretation, these findings merit further investigation.

In conclusion, we have demonstrated that preadipocyte differentiation within adipose tissue may be subject to both positive and negative regulators derived from AA metabolism. Furthermore, our data suggest that metabolites arising from LOX activity may be more potent, or present in greater abundance, than those resulting from COX activity. Studies are ongoing to identify which LOX products are synthesized by preadipocytes and adipocytes and to determine the physiological relevance of these observations.

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