# Arachidonic acid-dependent inhibition of adipocyte differentiation requires PKA activity and is associated with sustained expression of cyclooxygenases

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**Abstract** **Arachidonic acid inhibits adipocyte differentiation of 3T3-L1 cells via a prostaglandin synthesis-dependent pathway. Here we show that this inhibition requires the presence of a cAMP-elevating agent during the first two days of treatment. Suppression of protein kinase A activity by H-89 restored differentiation in the presence of arachidonic acid. Arachidonic acid treatment led to a prolonged activation of extracellular signal-regulated kinases 1 and 2 (ERK1/2), and suppression of ERK1/2 activity by the addition of U0126 rescued differentiation. Upon induction of differentiation, expression of cyclooxygenase-2 (COX-2) was transiently induced and then declined, whereas COX-1 expression declined gradually as differentiation progressed. Treatment with arachidonic acid led to sustained expression of COX-1 and COX-2. Omission of a cAMP-elevating agent or addition of H-89 or U0126 prevented sustained expression of COX-2. Unexpectedly, we observed that selective COX-1 or COX-2 inhibitors rescued adipocyte differentiation in the presence of arachidonic acid as effectively as did the nonselective COX-inhibitor indomethacin. De novo fatty acid synthesis, diacylglycerol acyltransferase (DGAT) activity, and triacylglycerol accumulation were repressed in cells treated with arachidonic acid. Indomethacin restored DGAT activity and triacylglycerol accumulation without restoring de novo fatty acid synthesis, resulting in an enhanced incorporation of arachidonic acid into cellular triacylglycerols.**—Petersen, R. K., C. Jørgensen, A. C. Rustan, L. Frøyland, K. Muller-Decker, G. Furstenberger, R. K. Berge, K. Kristiansen, and L. Madsen. **Arachidonic acid-dependent inhibition of adipocyte differentiation requires PKA activity and is associated with sustained expression of cyclooxygenases.** ***J. Lipid Res.*** **2003.** 44: **2320–2330.**

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Fatty acids derived from dietary fat act directly on preadipocytes to positively or negatively influence proliferation and differentiation. However, the underlying mechanisms dictating the opposing effects of distinct classes of dietary fatty acids are far from being fully understood. Whereas high-fat diets have been demonstrated to induce both hypertrophy and hyperplasia of adipose tissue in rats (1–5), diets rich in n-3 polyunsaturated fatty acids (n-3 PUFAs) prevent excessive adipose growth (2, 3, 6–10). A number of studies have demonstrated the beneficial effects of n-3 PUFAs on lipid-related disorders in humans (11–13), but concerns have been raised in regard to intake of n-6 PUFAs, insofar as they have been associated with an increased propensity to obesity (14, 15).

The major n-6 PUFA, arachidonic acid, has been identified as one of the adipogenic components of serum and is required for induction of differentiation of Ob1771 preadipose cells (16). The proadipogenic effect of arachidonic acid in this cell system is cyclooxygenase (COX)-dependent and mediated by prostacyclin (16–18). In sharp contrast, another arachidonic acid-derived metabolite, prostaglandin

Abbreviations: Akt/PKB, protein kinase B; aP2, adipocyte lipid binding protein; COX, cyclooxygenase; DGAT, diacylglycerol acyltransferase; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; ERK1/2, extracellular signal-regulated kinases 1 and 2; MAPK, mitogen-activated protein kinase; MDI, methylisobutylxanthine, dexamethasone, and insulin; MIX, methylisobutylxanthine; PPAR, peroxisome proliferator-activated receptor; SD, standard deviation; TFIIB, transcription factor IIB.

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$F_{2\alpha}$ ($PGF_{2\alpha}$), inhibits differentiation of primary preadipocytes (19, 20), 1246 cells (21), and 3T3-L1 cells (22–24). $PGF_{2\alpha}$ has been suggested to inhibit differentiation of 3T3-L1 preadipocytes through a $PGF_{2\alpha}$ receptor-mediated increase in intracellular calcium and increased DNA synthesis (22) and through activation of mitogen-activated protein kinase (MAPK), resulting in an inhibitory phosphorylation of peroxisome proliferator-activated receptor γ (PPARγ) (25).

The rate-limiting step in prostaglandin synthesis is catalyzed by COXs comprising the constitutive isoform, COX-1, and the inducible isoform, COX-2. COX-2 has been implicated in the regulation of body fat accumulation, as haploinsufficient mice are prone to develop obesity (26). Recently, 3T3-L1 preadipocytes were shown to express both COX isoforms, and both isoforms were suggested to negatively influence adipocyte differentiation (27).

The present study was undertaken to elucidate the molecular mechanisms by which arachidonic acid prevents adipocyte differentiation of 3T3-L1 preadipocytes and to investigate the role(s) of COX-1 and COX-2 in mediating the inhibitory effect of arachidonic acid. We show that arachidonic acid-mediated inhibition of differentiation requires both COX-1 and COX-2 activity. COX-2 expression was transiently induced upon induction of differentiation, and arachidonic acid treatment led to a sustained expression of COX-2. Addition of arachidonic acid led to a pronounced repression of de novo fatty acid synthesis, diacylglycerol acyltransferase (DGAT) activity, and triacylglycerol accumulation. Interestingly, indomethacin restored DGAT activity and triacylglycerol accumulation without fully restoring de novo fatty acid synthesis, suggesting a prostaglandin-independent repression of de novo fatty acid synthesis. Under these conditions, enhanced incorporation of arachidonic acid into cellular triacylglycerol was observed. We demonstrate that arachidonic acid-mediated inhibition of adipocyte differentiation and sustained expression of COX-2 require the inclusion of a cAMP-elevating agent in the adipogenic cocktail and are dependent on protein kinase A (PKA) and MAPK activity.

## MATERIALS AND METHODS

### Cell culture and differentiation

3T3-L1 cells were cultured to confluence in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) (28). Two days postconfluent (designated day 0), cells were induced to differentiate with DMEM supplemented with 10% fetal bovine serum (FBS), 1 μM dexamethasone (DEX) (Sigma), 0.5 mM methylisobutylxanthine (MIX) (Sigma), and 1 μg/ml insulin (Novo Nordisk A/S). Fatty acids (Sigma) and inhibitors were dissolved in DMSO and added when differentiation was induced. Cells not treated with fatty acids or inhibitors received similar volumes of DMSO. After 48 h, the media were replaced with DMEM supplemented with 10% FBS and 1 μg/ml insulin. The cells were subsequently refed every 48 h with DMEM supplemented with 10% FBS.

### Oil Red O staining

Staining of lipids by Oil Red O was performed as described previously (29).

### Whole-cell extracts and Western blot analysis

Whole-cell extracts, electrophoresis, blotting, visualization, and stripping of membranes were performed as described previously (29). The primary antibodies used were rabbit anti-p44/p42 MAPK and mouse anti-phospho-p44/p42 MAPK (Thr-202/Tyr-204) obtained from Cell Signaling Technology; rabbit anti-TFIIB, rabbit anti-PPARγ, rabbit anti-C/EBPβ, goat anti-COX-1, and goat anti-COX-2 antibodies obtained from Santa Cruz Biotechnology; and rabbit anti-ALBP/aP2 kindly provided by D. A. Bernlohr. Secondary antibodies were horseradish peroxidase-conjugated anti-mouse, anti-goat, or anti-rabbit antibodies obtained from DAKO.

### De novo fatty acid synthesis

Cells were incubated with [1(2)-$^{14}$C] acetic acid sodium salt (0.2 μCi/ml medium) (Amersham Pharmacia Biotech) for 4 h, harvested in water, and frozen. Fatty acids were extracted according to the procedure of Folch, Lees, and Sloane-Stanley (30) with minor modifications. Cells sonicated in 0.5 ml water were added drop-wise to 2.5 ml methanol with constant shaking, and 1.25 ml chloroform was then added. After shaking (300 rpm, 5 min) and centrifugation (2,000 *g*, 5 min), the pellet was dissolved in 2.5 ml methanol and 3.75 ml chloroform. After a second shaking (300 rpm, 5 min) and centrifugation (2,000 *g*, 5 min), the supernatants were collected, and 4 ml water was added. Separation was allowed to proceed overnight at −20°C. After centrifugation (2,000 *g*, 5 min) at room temperature, the upper phase was removed and the lower phase dried under $N_2$, at 35°C. The lipids were hydrolyzed in 2 ml 0.5 M KOH in ethanol:water (9:1; v/v) for 60 min at 80°C, followed by acidification by 0.35 ml 4 M HCl. The fatty acids were extracted twice with 3 ml hexane, dried under $N_2$, at 35°C, dissolved in 8 ml scintillation liquid, and quantified by scintillation counting.

### Cellular levels of triacylglycerols

Cells grown in 6-well plates were harvested in 1 ml water and frozen. The cells were sonicated, and the cellular levels of triacylglycerols were measured on an AXON Byer spectrophotometer using the TRINDER reaction kit from bioMèrieux.

### DGAT activity

Cells grown in 15 cm plates were washed and harvested in 1 ml homogenzation buffer [0.25 M sucrose, 10 mM HEPES (pH 7.4) and 2 mM EDTA] followed by homogenization by repeated forcing of the cell suspensions through a ball bearing homogenizer, and a postnuclear fraction was prepared as described previously (31). DGAT activity was measured in the postnuclear fractions as originally described by Coleman and Bell (32) with modifications as described by Rustan et al. (33) using oleoyl-CoA and 1,2-di[1-$^{14}$C]oleoylglycerol as substrates.

### Arachidonic acid incorporation into glycerolipids

Cells grown in 6-well plates were washed twice with PBS. The cells were subsequently incubated with 2 ml serum-free medium containing 10 μM U-[$^{14}$C]arachidonic acid for 4 h. The incubations were terminated by cooling on ice, and the medium was subsequently centrifuged at 600 *g* for 5 min. $^{14}CO_2$ was trapped from cell-free medium in sealed tubes as described previously (31). The cells were washed twice with PBS and harvested in 2 ml PBS. The cell suspensions were centrifuged and resuspended in 0.5 ml distilled water and frozen. The lipids were extracted from the cell suspensions (30), dissolved in *n*-hexane, and separated by TLC on silica gel plates using hexane-diethyl ether-acetic acid (65:35:1; v/v/v). The bands were visualized by iodine vapor, cut into pieces, and assayed for radioactivity by scintillation counting.

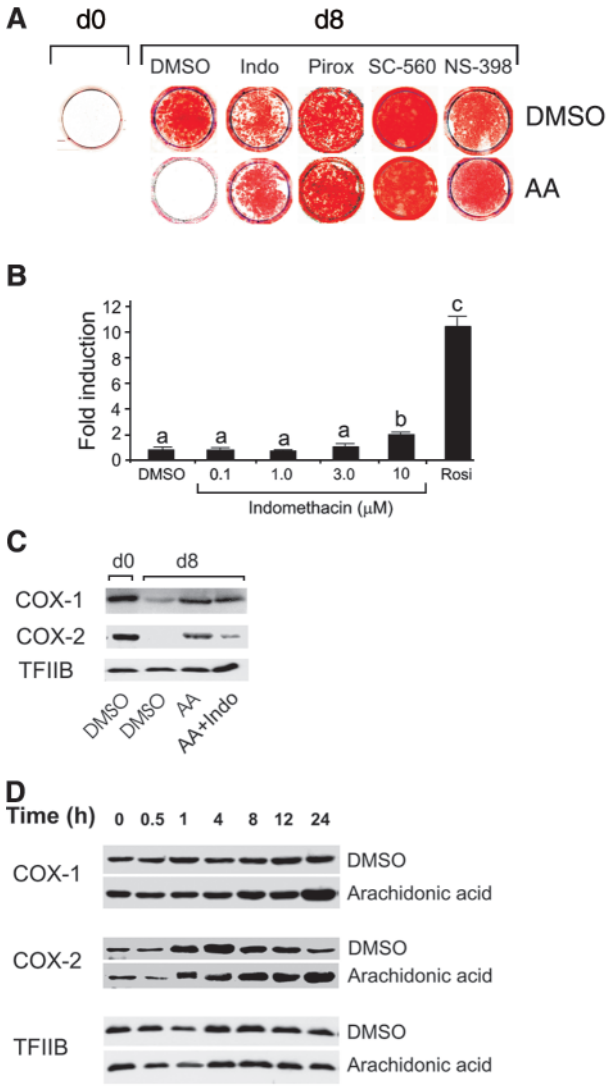


**Fig. 1.** Arachidonic acid-mediated inhibition of adipocyte differentiation is dependent on cyclooxygenase-1 (COX-1) and COX-2 activity. 3T3-L1 cells were induced to differentiate at 2 days postconfluence (designated day 0) with DMEM containing 10% fetal bovine serum (FBS), 1 μM dexamethasone (DEX), 0.5 mM methylisobutylxanthine (MIX), and 1 μg/ml insulin. After 48 h, the cells were refed with DMEM containing 10% fetal calf serum (FCS) and 1 μg/ml insulin. From day 4, medium consisted of DMEM with 10% FBS and was changed every second day. Arachidonic acid and/or inhibitors, all dissolved in DMSO, were added as indicated when cells were induced on day 0 and were present throughout the differentiation period. When added, the final concentrations were: 100 μM arachidonic acid (AA), 1 μM indomethacin (Indo), 5 μM piroxicam (Pirox), 50 nM SC-560, and 5 μM NS-398. The final concentration of DMSO was kept at 0.1% in all experiments. A: The cells were stained with Oil Red O and photographed on day 0 and day 8. B: 3T3-L1 cells were transiently transfected with the $UAS_{GAL}$x4-TK-luc reporter, a vector expressing Gal4-DBD-mPPARγ-LBD, and a CMV-β-galactosidase vector as described in Materials and Methods. The cells were incubated for 24 h in the absence or presence of increasing concentrations of indomethacin dissolved in DMSO. Rosiglitazone (Rosi) was used as positive control. Data are presented as luciferase activity normalized to β-galactosidase ± standard deviation (SD). Different letters (a, b, c) indicate values that differ significantly ($P < 0.05$). C: Whole-cell extracts were prepared at the time of induction and on day 8 after induction and were analyzed for expression of COX-1 and COX-2 by Western blotting. Antibody recognizing TFIIB (transcription factor IIB) was used as control for equal loading. D: Whole-cell extracts were prepared at the time of induction and 0.5, 1, 4, 8, 12, and 24 h after induction and were analyzed for expression of COX-1 and COX-2 by Western blotting. Antibody recognizing TFIIB was used as control for equal loading.

### Transient transfection

The UASx4-TK-luc reporter, containing four Gal4-responsive elements, was kindly provided by Ronald M. Evans. The pcDNA1-GAL4-mPPARγ(LBD) encoding the Gal4-DBD-mPPARγ-LBD fusion has been described previously(28). The CMV-β-galactosidase plasmid used for normalization was from Clontech. 3T3-L1 cells were plated in 12-well plates 12 h before transfection. One hour prior to transfection, the medium was changed, and each well was transfected with 400 ng $UAS_{GAL}$x4-TK-luc reporter, 200 ng Gal4-DBD-mPPARγ-LBD, and 50 ng CMV-β-galactosidase using the calcium-phosphate protocol. Five hours later, fresh medium containing resin-charcoal-stripped 10% FCS and indomethacin dissolved in DMSO, or DMSO alone, was added. After 24 h, the cells were harvested. Luciferase and β-galactosidase activities were measured in a Berthold MicroLumat LB96P luminometer using a commercial kit (Galacto-Light, Applied Biosystems, Foster City, CA). Luciferase values were normalized to β-galactosidase values.

### Statistical analysis and presentation of data

All experiments were performed in duplicate or triplicate and were repeated two to five times. The data presented in bars represent means ± standard deviation of triplicates in a representative experiment. Where relevant, a two-tailed Student's *t*-test was applied for equality of means at the significance level of $P < 0.05$.

## RESULTS

### Arachidonic acid-dependent inhibition of adipocyte differentiation requires both COX-1 and COX-2 activity

Arachidonic acid-mediated inhibition of differentiation of 3T3-L1 cells is reversed by addition of COX inhibitors, suggesting that the inhibitory effect of arachidonic acid is mediated by prostaglandins (23). Both the constitutively expressed COX-1 and the inducible COX-2 are expressed in undifferentiated 3T3-L1 preadipocytes (27), but the roles of the individual COX isoforms in adipogenesis have not been elucidated.

Here we induced 3T3-L1 cells to differentiate by ny linje methylisobutylxanthine, dexamethasone, and insulin (MDI) in the presence of arachidonic acid and the selective COX-1 inhibitors piroxicam and SC-560, the selective COX-2 inhibitor NS-398, as well as the nonselective COX inhibitor indomethacin. Confirming previous results (23), administration of the nonselective COX inhibitor indomethacin fully rescued adipocyte differentiation of 3T3-L1 cells in the presence of arachidonic acid (**Fig. 1A**). Surprisingly, however, administration of the selective COX-1 inhibitors piroxicam and SC-560, as well as the selective COX-2 inhibitor NS-398, also rescued differentiation in the presence of arachidonic acid (Fig. 1A). Inhibition of COX activity has been reported to stimulate differentiation of

3T3-L1 cells (3, 27, 34, 35), but the biological significance of these observations is obscured by the fact that several COX inhibitors, including indomethacin, are also PPARγ agonists, with reported $EC_{50}$ values in the micromolar range (36, 37). In our cell system, indomethacin at 1 μM did not enhance PPARγ-dependent transactivation (Fig. 1B). Furthermore, the selective inhibitors of COX-1 and -2, piroxicam, SC-560, and NS-398, did not enhance PPARγ-dependent transactivation at the concentrations used in this study (results not shown) and did not affect MDI-induced differentiation (Fig. 1A).

The finding that piroxicam, SC-560, and NS-398 rescued adipocyte differentiation in the presence of arachidonic acid suggests that arachidonic acid-dependent inhibition requires both COX-1 and COX-2 activity. To determine whether constitutive COX activity was necessary for expression of one or the other isoform, the expression of COX-1 and COX-2 was determined by Western blotting of 3T3-L1 cells induced to differentiate by MDI in the presence of arachidonic acid and the nonselective COX inhibitor indomethacin. As reported (27), we found that both COX-1 and COX-2 were expressed in undifferentiated 3T3-L1 cells at day 0. In the differentiated state, COX-1 expression was reduced but still clearly detectable, whereas expression of COX-2 was barely discernible in day 8 adipocytes (Fig. 1C). Both COX-1 and COX-2 expression remained high in cells treated with arachidonic acid (Fig. 1C). The sustained expression of COX-1 in arachidonic acid-treated cells is not solely a result of impaired differentiation, inasmuch as COX-1 expression remained high even in the presence of indomethacin (Fig. 1C). Thus, prostaglandin synthesis is not a prerequisite for the sustained COX-1 expression in cells treated with arachidonic acid. In contrast, indomethacin very strongly reduced COX-2 expression in cells treated with arachidonic acid, indicating either that prostaglandins are necessary for maintaining COX-2 expression or that COX-2 expression is downregulated in response to adipocyte differentiation (Fig. 1C).

To follow the immediate changes in COX expression upon induction of differentiation, 3T3-L1 cells were induced to differentiate by MDI and cells were harvested at several time points after induction during the first 24 h. Figure 1D shows that COX-2 expression is increased 1.0 h after induction in both the presence and the absence of arachidonic acid. After 12 h, COX-2 expression declined in DMSO-treated cells, but expression was maintained in cells treated with arachidonic acid (Fig. 1D).

The finding that both selective COX-1 and selective COX-2 inhibitors rescued arachidonic acid-dependent inhibition of adipocyte differentiation indicates that products from both COX isoforms are necessary for mediating the effect of arachidonic acid. To investigate this possibility, 3T3-L1 cells were induced to differentiate by MDI in the presence of arachidonic acid and the selective COX-1 inhibitors piroxicam and SC-560, the selective COX-2 inhibitor NS-398, as well as the nonselective COX inhibitor indomethacin. The cells were harvested after 3 and 6 h, and the levels of prostaglandins were measured in the supernatants. Interestingly, we found that production of the inhibitory $PGE_2$ in arachidonic acid-treated cells was reduced by both the COX-1 and COX-2 selective inhibitors (results not shown).

**Arachidonic acid regulates triacylglycerol accumulation at the level of de novo fatty acid synthesis and DGAT activity**

During adipocyte differentiation, triacylglycerol accumulation is dependent on the overall supply of fatty acid from uptake or de novo synthesis and the rate of triacylglycerol synthesis, in which the last and rate-determining step is catalyzed by DGAT1. To determine how arachidonic acid affects these processes quantitatively, we first measured triacylglycerol accumulation in vehicle-treated cells (DMSO) and cells treated with arachidonic acid in the absence or presence of different inhibitors affecting prostaglandin synthesis. **Figure 2A** shows that the nonselective COX inhibitor indomethacin, as well as the selective COX-1 and COX-2 inhibitors piroxicam, SC-560, and NS-398, respectively, completely rescued triacylglycerol accumulation of arachidonic acid-treated cells. However, the rate of de novo fatty acid synthesis was only partially restored, reaching ~50% of the level in cells differentiated in the absence of arachidonic acid (Fig. 2B). This suggests that arachidonic acid reduces the rate of de novo fatty acid synthesis, at least partially, in a prostaglandin-independent manner. We hypothesized that the inhibitory action of arachidonic acid on fatty acid synthesis was related to its polyunsaturated nature, and therefore, we compared the effects of arachidonic acid with those of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), oleic acid, and palmitic acid on triacylglycerol accumulation and de novo fatty acid synthesis in MDI-treated 3T3-L1 cells. The saturated palmitic acid and the monounsaturated oleic acid had no effect on triacylglycerol accumulation. In contrast, the two n-3 PUFAs, EPA and DHA, reduced triacylglycerol accumulation by 15% and 25%, respectively, and this reduction was not prevented by indomethacin (Fig. 2C). Similarly, palmitic acid and oleic acid did not affect de novo fatty acid synthesis, whereas EPA and DHA reduced de novo fatty acid synthesis in proportion to the reduction in triacylglycerol accumulation. As predicted, this reduction was not prevented by indomethacin (Fig. 2D), suggesting that polyunsaturated fatty acids per se are able to diminish the rate of de novo fatty acid synthesis.

The finding that COX inhibitors completely rescued arachidonic acid-dependent inhibition of triacylglycerol accumulation but only partially restored de novo fatty acid synthesis suggests either that de novo fatty acid synthesis is not limiting under these conditions or that exogenous fatty acids, especially arachidonic acid, secure a sufficient supply for triacylglycerol synthesis. In this case, the proportion of arachidonic acid in the cellular triacylglycerol pool would be predicted to increase. To test this, we induced 3T3-L1 cells to differentiate by MDI in the absence and presence of arachidonic acid and indomethacin. On day 5 of the differentiation process, the cells were incubated with [$^{14}$C]arachidonic acid for 4 h, and the amount

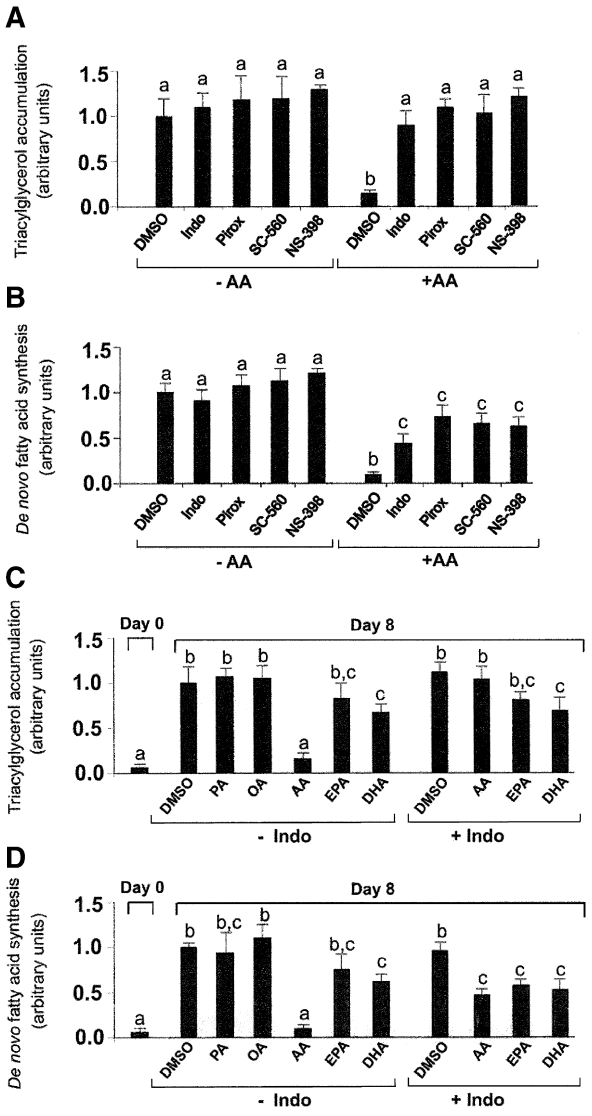


**Fig. 2.** Polyunsaturated fatty acids inhibit adipocyte differentiation. 3T3-L1 cells were induced to differentiate and were treated with arachidonic acid and/or inhibitors as described in Fig. 1. On day 8, the levels of triacylglycerols were measured in whole-cell extracts (A) or the cells were incubated with [$^{14}$C]acetic acid for 4 h (B). The cells were harvested, and labeled fatty acids were extracted and quantitated by scintillation counting. One hundred micromoles palmitic acid (PA), oleic acid (OA), arachidonic acid (AA), eicosapentaenoic acid (EPA), or docosahexaenoic acid (DHA) were dissolved in DMSO and added when differentiation was induced on day 0 and were present throughout the differentiation period. The levels of triacylglycerols were measured in whole-cell extracts (C) or the cells were incubated with [$^{14}$C]acetic acid for 4 h (D). The cells were harvested, and labeled fatty acids were extracted and quantitated by scintillation counting. Different letters (a, b, c) indicate values that differ significantly ($P < 0.05$).

of arachidonic acid used for β-oxidation, the concentration of free arachidonic acid in the cells, as well as the incorporation of arachidonic acid into the major classes of lipids were determined.

On the basis of these data, we calculated the percentage of added [$^{14}$C]arachidonic acid used for β-oxidation and the relative incorporation of [$^{14}$C]arachidonic acid into phospholipids, diacylglycerols, and triacylglycerols (**Fig. 3**). Less than 1% of the added arachidonic acid was oxidized to $CO_2$ (Fig. 3A), and generally, the majority of [$^{14}$C]arachidonic acid was incorporated into phospholipids (Fig. 3B). Figure 3B shows that the percentage of [$^{14}$C]arachidonic acid incorporated into phospholipids did not differ between DMSO-treated cells and cells grown in the presence of arachidonic acid. Administration of indomethacin resulted in a slight but insignificant

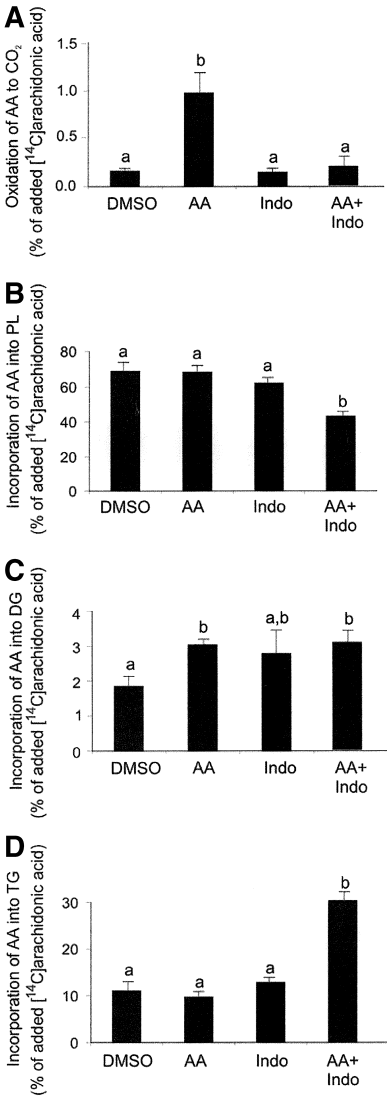


**Fig. 3.** Incorporation of [U-$^{14}$C]arachidonic acid into cellular lipids. 3T3-L1 cells were induced to differentiate as described in Fig. 1 in the absence or presence of 100 μM arachidonic acid (AA) and/or 1 μM indomethacin (Indo) dissolved in DMSO. Arachidonic acid and inhibitors were added when differentiation was induced on day 0. On day 5, the cells were washed with PBS and incubated with 10 μM [U-$^{14}$C]arachidonic acid for 4 h. $^{14}CO_2$ was trapped from cell-free media in sealed tubes (A). The lipids were extracted from the cells and separated on TLC. Bands were visualized by iodine vapor, cut into pieces, and counted in a scintillation counter. The relative incorporation of [$^{14}$C]arachidonic acid into phospholipids (PL) (B), diacylglycerols (DG) (C), and triacylglycerols (TG) (D) was calculated. Different letters (a, b) indicate values that differ significantly ($P < 0.05$).

decrease in the percentage of incorporated [$^{14}$C]arachidonic acid. Of note, the simultaneous treatment with arachidonic acid and indomethacin significantly reduced the relative amount of [$^{14}$C]arachidonic acid incorporated into phospholipids (Fig. 3B). Incorporation of [$^{14}$C]arachidonic acid into diacylglycerols was increased by ~50% in cells incubated with arachidonic acid and in cells treated with both arachidonic acid and indomethacin (Fig. 3C). It appeared that treatment with indomethacin alone led to an increased incorporation of [$^{14}$C]arachidonic acid. However, the difference between indomethacin-treated cells and control cells (or cells treated with arachidonic acid in the absence or presence of indomethacin) was not statistically significant (Fig. 3C). The relative incorporation of [$^{14}$C]arachidonic acid into triacylglycerols was similar in vehicle-treated cells and cells incubated with arachidonic acid. Furthermore, indomethacin alone only slightly increased the percentage of [$^{14}$C]arachidonic acid incorporated into triacylglycerols, and this increase was not statistically significant (Fig. 3D).

In contrast, the relative incorporation of [$^{14}$C]arachidonic acid into triacylglycerols in cells subjected to combined treatment with arachidonic acid and indomethacin increased dramatically (Fig. 3D). Because the relative incorporation of [$^{14}$C]arachidonic acid into phospholipids decreased (Fig. 3B), we conclude that there is a selective channeling of arachidonic acid into triacylglycerols in cells incubated with arachidonic acid in the presence of indomethacin. These results indicated that arachidonic acid inhibited triacylglycerol synthesis in a prostaglandin-dependent manner. To test this hypothesis, we induced 3T3-L1 cells to differentiate in the absence and presence of arachidonic acid and indomethacin and determined the activity of DGAT using [$^{14}$C]diacylglycerol and oleoyl-CoA as substrates. By day 5, DGAT activity in MDI-treated cells had increased more than 9-fold compared with day 0. As predicted, DGAT activity was severely reduced in cells treated with arachidonic acid, and the arachidonic acid-dependent reduction of DGAT activity was rescued by indomethacin (**Fig. 4**).

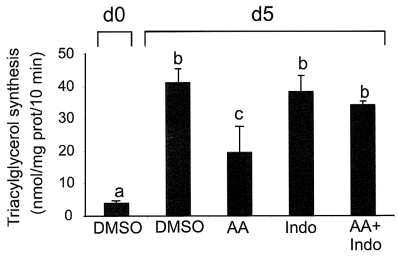


**Fig. 4.** Indomethacin rescues arachidonic acid-dependent inhibition of diacylglycerol acyltransferase (DGAT) activity. 3T3-L1 cells were induced to differentiate as described in Fig. 1 in the absence or presence of 100 μM arachidonic acid (AA) and/or 1 μM indomethacin (Indo) dissolved in DMSO. Arachidonic acid and indomethacin were added when differentiation was induced at day 0. On day 5, the cells were harvested and homogenized. DGAT activity was measured in the postnuclear fraction using oleoyl-CoA and 1,2-di[1-$^{14}$C]oleoylglycerol as substrates. Different letters (a, b, c) indicate values that differ significantly ($P < 0.05$).

### Arachidonic acid-dependent inhibition of adipocyte differentiation and sustained COX-2 expression require agents that elevate cAMP levels

The inhibitory effect of arachidonic acid on differentiation of 3T3-L1 preadipocytes is well documented (23, 24), but arachidonic acid has also been reported to stimulate differentiation of Ob17 (38), Ob1771 (16, 18), and 3T3-F442A cells (16). Ob1771 and 3T3-F442A cells are induced to differentiate in media containing no MIX or other cAMP-elevating agents (16–18). We therefore asked if the inhibitory action of arachidonic acid was dependent on MIX or other agents that increase the level of cAMP, and whether arachidonic acid was able to stimulate differentiation of 3T3-L1 cells when MIX was absent.

3T3-L1 preadipocytes were treated with different combinations of the adipogenic inducers DEX, MIX, and insulin in the absence or presence of arachidonic acid. The combinations MIX plus insulin, and DEX plus MIX and insulin strongly induced adipocyte differentiation as visualized by Oil Red O staining. MIX plus DEX, and DEX plus insulin induced adipocyte differentiation less strongly, as reported previously (39) (**Fig. 5A**). Importantly, arachidonic acid prevented adipocyte differentiation in all combinations in which MIX was included. In contrast, arachidonic acid strongly induced adipocyte differentiation in cells treated with only DEX plus insulin, and a slight stimulation was also observed in cells treated with DEX plus arachidonic acid (Fig. 5A). The patterns of triacylglycerol accumulation were paralleled by induction of PPARγ1, PPARγ2, and adipocyte lipid binding protein (aP2), as determined by Western blotting. Furthermore, high levels of COX-2 expression were observed in cells treated with arachidonic acid in the presence of MIX (Fig. 5B). Inclusion of either forskolin (Fig. 5C) or 100 μM 8-(4-chlorophenylthio)-cAMP (not shown) abolished the ability of arachidonic acid to induce differentiation in the presence of DEX and insulin, corroborating the notion that the inhibitory effect of arachidonic acid requires elevated cAMP levels.

### Arachidonic acid-dependent inhibition of adipocyte differentiation requires PKA and MAPK activity

The finding that arachidonic acid-induced inhibition of adipocyte differentiation is dependent on a cAMP-elevating agent suggests that arachidonic acid mediates its effect by a PKA-sensitive pathway. As anticipated, inclusion of the PKA inhibitor H-89 rescued the differentiation of arachidonic acid-treated cells, as determined by Oil Red O staining of lipids and measurements of triacylglycerol levels and de novo fatty acid synthesis (**Fig. 6A–C**). It is noteworthy that addition of H-89 did not impair, but rather, slightly enhanced adipocyte differentiation in vehicle-treated cells (Fig. 6A–C).

Activation of cAMP-dependent PKA has been demonstrated to enhance MAPK activity and phosphorylation of PPARγ (40). We therefore predicted that inclusion of the MEK inhibitor U0126 might rescue arachidonic acid-mediated inhibition of adipocyte differentiation. As

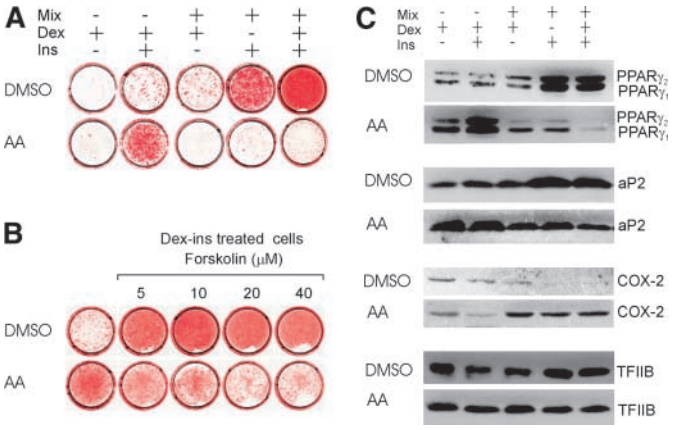


**Fig. 5.** Arachidonic acid-mediated inhibition of adipocyte differentiation is dependent on the addition of a cAMP-elevating agent. 3T3-L1 cells were induced to differentiate at day 2 postconfluence with DMEM containing 10% FBS and different combinations of DEX, insulin, MIX, and forskolin, as indicated, in the absence or presence of 100 μM arachidonic acid. After 48 h, the cells were refed with DMEM containing 10% FCS and 1 μg/ml insulin if present from day 0. A, B: The cells were stained with Oil Red O and photographed on day 8. C: Whole-cell extracts were prepared on day 8 and analyzed for the expression of peroxisome proliferator-activated receptor γ (PPARγ), adipocyte lipid binding protein (aP2), and COX-2 by Western blotting. Antibody recognizing TFIIB was used as control for equal loading.

shown in Fig. 6, this was indeed the case. In the presence of U0126, mitotic clonal expansion is inhibited, and hence the number of differentiated cells per dish was reduced. However, Oil Red O staining demonstrated that differentiation per se was unaffected by arachidonic acid in the presence of U0126 (Fig. 6A). In addition, when triacylglycerol accumulation and de novo fatty acid synthesis were corrected for differences in the number of cells, and hence amounts of protein, per dish in the U0126-treated cells, it was obvious that U0126 completely restored both triacylglycerol accumulation and de novo fatty acid synthesis (Fig. 6B, C). Western blotting demonstrated that inclusion of MEK and PKA inhibitors restored expression of PPARγ and aP2 in the presence of arachidonic acid, and interestingly, the sustained expression of COX-2 by arachidonic acid was also prevented by both U0126 and H-89 (Fig. 6D).

To examine whether treatment with arachidonic acid affected MAPK activation observed upon administration of adipogenic inducers, activation of extracellular signal-regulated kinase 1and 2 (ERK1/2) was assessed by Western blotting using antibodies recognizing the phosphorylated (active) forms of ERK1/2. Antibodies recognizing total ERK1/2 were used as control for equal loading. **Figure 7A** shows that ERK1 and ERK2 were strongly activated 0.5 h and 1.0 h after induction of differentiation, but the levels of activated ERK1 and ERK2 were higher in arachidonic acid-treated cells, and furthermore, higher levels of ERK1/2 activity were maintained for a more prolonged period in cells incubated with arachidonic acid (Fig. 7A). After 12 h, no active ERK1 and ERK2 were observed in control cells, whereas ERK2 activity was still detectable in arachidonic acid-treated cells (Fig. 7A). As expected, the MEK inhibitor U0126 prevented induction of ERK activity, whereas indomethacin and H-89 did not diminish the augmented ERK1/2 activity in arachidonic acid-treated cells (Fig. 7B).

It has been suggested that induction of PPARγ2 but not PPARγ1 is dependent on the MIX-inducible CCAAT/enhancer binding protein β (C/EBPβ) (39). Our finding that low expression of PPARγ2 but not PPARγ1 in the presence of arachidonic acid is MIX dependent suggested the possibility that arachidonic acid mediated its effect on PPARγ2 expression by altering the expression of C/EBPβ. **Figure 8** demonstrates that the ratio between the transactivating 34 kDa isoform (LAP) and the inhibitory 20 kDa isoform (LIP) is shifted toward LIP in the presence of arachidonic acid in MDI-treated cells. Ectopic expression of the LIP isoform of C/EBPβ has been demonstrated to inhibit induction of PPARγ2 (39), and hence induction of LIP by arachidonic acid may contribute to its inhibitory effect on adipocyte differentiation.

## DISCUSSION

The role of COX and prostaglandins in the regulation of adipocyte differentiation is complex, because different classes of prostaglandins exert opposing effects on adipocyte differentiation. Thus, $PGI_2$ has been reported to promote adipocyte differentiation (17, 41), whereas $PGF_{2\alpha}$ inhibits differentiation via a p42/p44 MAPK-mediated phosphorylation of PPARγ (25, 42). In addition, $PGE_2$ has also been demonstrated to be a potent inhibitor of adipocyte differentiation (23). Here we report that production of the inhibitory $PGE_2$ is reduced by selective COX-1 as well as selective COX-2 inhibitors, providing, at least in part, an explanation of how either type of inhibitor might rescue adipocyte differentiation in the presence of arachidonic acid.

In this study, we demonstrate that COX-2 expression is transiently upregulated during the first hour after induction of differentiation by MDI and then declines during the differentiation process. When adipocyte differentiation is inhibited by arachidonic acid, COX-2 expression is sustained. The finding that inclusion of the nonselective COX inhibitor indomethacin rescues arachidonic acid-mediated inhibition of adipocyte differentiation and partly prevents sustained COX-2 expression indicates that sustained elevated COX-2 expression correlates with impaired differentiation. The PKA inhibitor H-89 was able to prevent the sustained COX-2 expression observed in arachidonic acid-treated cells. A cAMP-responsive element in the COX-2 promoter has been shown to be essential for induction of COX-2 expression in mouse fibroblasts and activated mast cells (43–45), and forskolin and dibutryl-cAMP have been shown to induce COX-2 (and COX-1) expression in cultured mouse keratinocytes (46).

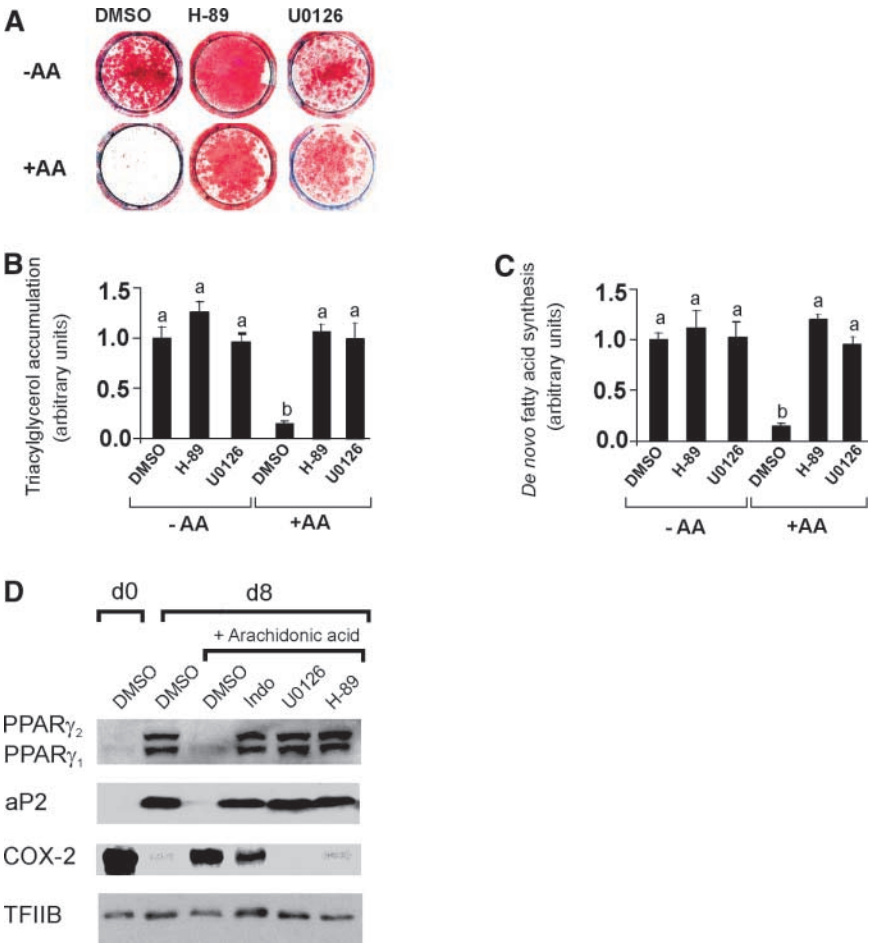


**Fig. 6.** Arachidonic acid-mediated inhibition of adipocyte differentiation requires PKA and mitogen-activated protein kinase (MAPK) activity. 3T3-L1 cells were induced to differentiate as described in Fig. 1. One hundred micromoles arachidonic acid (AA) and/or inhibitors (10 μM H-89 or 10 μM U0126) dissolved in DMSO were added when differentiation was induced at day 0 and were present throughout the differentiation period. On day 8, the cells were stained with Oil Red O and photographed (A), the levels of triacylglycerols were measured in whole-cell extracts (B), or the cells were incubated with U-$^{14}$C-labeled acetic acid for 4 h for analysis of de novo fatty acid synthesis (C). Results are presented as mean ± SD. Different letters (a, b) indicate values that differ significantly ($P < 0.05$). D: One hundred micromoles arachidonic acid and/or inhibitors [1 μM indomethacin (indo), 10 μM H-89, or 10μM U0126] dissolved in DMSO were added when differentiation was induced at day 0 and were present throughout the differentiation period. Whole-cell extracts were prepared on day 8 and analyzed for expression of PPARγ, aP2, and COX-2 by Western blotting. Antibody recognizing TFIIB was used as control for equal loading.

Here we demonstrate that sustained COX-2 expression in arachidonic acid-treated 3T3-L1 cells requires the addition of a cAMP-elevating agent and is dependent on PKA-mediated signaling. In contrast, we observed, surprisingly, that the stimulatory action of MIX on adipocyte differentiation in the absence of arachidonic acid was not abolished by H-89. This would indicate that elevated cAMP levels stimulate differentiation in a PKA-independent manner. One possibility would be that cAMP, via activation of an Epac1-Rap1-Rac-dependent pathway (47, 48), negatively regulates Rho activity and hence diminishes the activity of Rho kinase(s), recently shown to be involved in the switching between adipogenesis and myogenesis (49). It should also be noted that cAMP-dependent activation of Epac1 may lead to enhanced Akt/PKB activation (50).

It is generally agreed that a transient upregulation of ERK1/2 MAPK activity is a prerequisite for clonal expansion and promotes adipogenesis (51–53), whereas sustained activation of the ERK1/2 has been demonstrated to inhibit adipogenesis (54–57). We show that addition of arachidonic acid enhances ERK1/2 activation following induction of adipocyte differentiation by MDI and prolongs the period during which ERK2, in particular, remains in the active state. Thus, it is likely that the more sustained activation of ERK1/2 in arachidonic acid-treated cells, in part via phosphorylation of PPARγ, contributes to the observed impairment of adipocyte differentiation. We found that administration of the MEK inhibitor U0126 restored adipogenesis in the presence of arachidonic acid. Inspection of the Oil Red O-stained dishes demonstrated that the overall accumulation of triacylglycerols was diminished in cells treated with U0126 but that the triacyl-

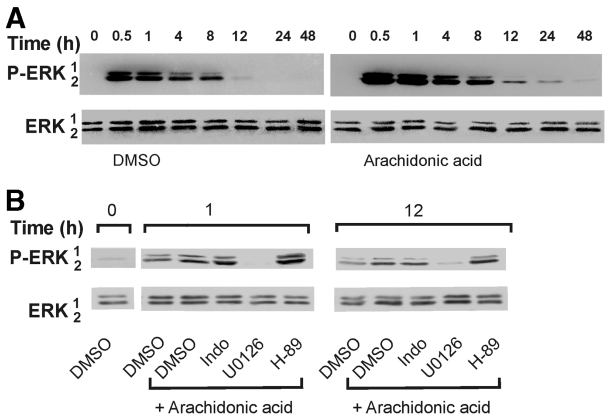


**Fig. 7.** Arachidonic acid stimulates MAPK activity. 3T3-L1 cells were induced to differentiate as described in Fig. 1. A: One hundred micromoles arachidonic acid dissolved in DMSO or DMSO alone were added when differentiation was induced. Whole-cell extracts were prepared at the time of induction and 0.5, 1, 4, 8, 12, 24, and 48 h after induction and analyzed for the presence of active extracellular signal-regulated kinase 1/2 (ERK1/2) or total ERK1/2 by Western blotting. B: One hundred micromoles arachidonic acid and/or inhibitors [1 μM indomethacin (Indo), 10 μM U0126, or 10 μM H-89] were dissolved in DMSO and were added when differentiation was induced. Whole-cell extracts were prepared at the time of induction and 1 and 12 h after induction and analyzed for the presence of active ERK1/2 or total ERK1/2 by Western blotting.

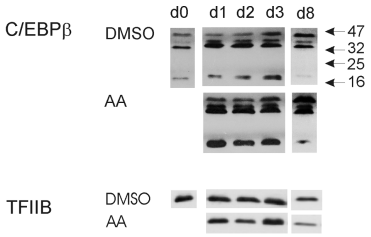


**Fig. 8.** Arachidonic acid induces the expression of the 20 kDa isoform of C/EBPβ. 3T3-L1 cells were induced to differentiate as described in Fig. 1. One hundred micromoles arachidonic acid (AA) dissolved in DMSO was added when differentiation was induced on day 0 and was present throughout the differentiation period. Whole-cell extracts were prepared at the time of induction and 1, 2, 3, and 8 days after induction and were analyzed for expression of C/EBPβ isoforms by Western blotting. Antibody recognizing TFIIB was used as control for equal loading.

glycerol accumulation was unaffected by arachidonic acid. Inhibition of the MEK-MAPK pathway impairs clonal expansion. Thorough research by Tang, Otto, and Lane (58) has provided evidence that clonal expansion is an integral part of the differentiation process and that adipocyte differentiation is prevented in the absence of clonal expansion. However, it has also been reported that clonal expansion, at least under certain conditions, is dispensable (53, 54, 57). Our finding is compatible with this view, although we cannot rule out the possibility that some ERK1/2 activity remains in the U0126-treated cells, allowing some degree of clonal expansion. However, it is important to note that the same degree of differentiation was achieved in U0126-treated cells regardless of the presence or absence of arachidonic acid.

Interestingly, the MEK inhibitor U0126 prevented sustained COX-2 expression, indicating that both PKA- and ERK1/2-dependent pathways are involved in the arachidonic acid-dependent upregulation of COX-2 expression, as in cardiomyocytes (59). Induction of COX-2 in NIH-3T3 cells by platelet-derived growth factor or serum requires activation of an Ras/Raf1/MEK/ERK pathway (60), and ERK1/2s are required for COX-2 induction by v-src in the same cells (44). Thus, sustained COX-2 activation and prolonged synthesis of antiadipogenic prostaglandins induced by arachidonic acid in the presence of MIX might be mediated by ERK1/2 in a similar manner in 3T3-L1 cells.

It has been demonstrated that PKA-dependent activation of ERK1/2 occurs in certain cell types (40). However, inclusion of the PKA inhibitor H-89 did not prevent the enhanced activation of ERK1/2 in arachidonic acid-treated 3T3-L1 cells. Like sustained COX expression, general inhibition of adipocyte differentiation by arachidonic acid was found to be dependent upon the addition of cAMP-elevating agents. Interestingly, we found that arachidonic acid exerted its effect at two levels: a repression of de novo fatty acid synthesis, which was only partly rescued by COX inhibitors, and a repression of DGAT activity, which was rescued by COX inhibitors. In the presence of indomethacin, arachidonic acid decreased de novo fatty acid synthesis to the same extent as EPA and DHA, whereas the rate of de novo fatty acid synthesis was unaffected by oleic and palmitic acid, suggesting that this mode of inhibition of de novo fatty acid synthesis reflects the unsaturated nature of the fatty acids. Because addition of arachidonic acid led to an enhanced activation of ERK1/2, and the MEK inhibitor U0126 restored de novo fatty acid synthesis in arachidonic acid treated-cells, it will be of interest to determine whether the apparently generalized PUFA effect on de novo fatty acid synthesis involves ERK1/2-dependent signaling. Formally, the repressive effect of PUFAs might be dependent on the activity of lipoxygenases. However, inhibition of lipoxygenase activity prevents adipocyte differentiation (28), and hence, the use of lipoxygenase inhibitors would be noninformative.

When 3T3-L1 cells are induced to differentiate in the presence of arachidonic acid, both PPARγ1 and PPARγ2 are expressed at very low levels. The low expression of PPARγ2 in the presence of arachidonic acid is MIX dependent. It has been suggested that induction of PPARγ2 is controlled in part via the MIX-inducible C/EBPβ (39). In addition, ectopic expression of the LIP isoform of C/EBPβ, which lacks the transactivation domain, inhibits induction of PPARγ2 (39). In this context, it is noteworthy that the addition of arachidonic acid shifts the ratio between LIP and LAP toward LIP, indicating that arachidonic acid-induced expression of the C/EBPβ isoform LIP contributes to the inhibitory effect of arachidonic acid on adipocyte differentiation.

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