Selective cyclo-oxygenase-2 inhibitors impair adipocyte differentiation through inhibition of the clonal expansion phase

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Abstract Selective cyclo-oxygenase-2 (COX-2) inhibitors are nonsteroidal antiinflammatory drugs used in the management of inflammatory diseases. We demonstrate here that inhibition of the COX-2 enzyme impairs adipocyte differentiation. The inhibition of adipogenesis occurs in the early clonal expansion phase. In particular, COX-2 inhibition limits cell cycle reentry required before terminal adipocyte differentiation. This inhibition of adipogenesis is independent of the production of the peroxisome proliferator activated receptor γ ligand prostaglandin J2, but dependent on the production of proliferative prostaglandins, such as prostaglandin E2. Modulation of the activity of the COX-2 enzyme via COX-2 selective inhibitors might open up new perspectives in the control of obesity and related metabolic diseases.—Fajas, L., S. Miard, M. R. Briggs, and J. Auwerx. **Selective cyclo-oxygenase-2 inhibitors impair adipocyte differentiation through inhibition of the clonal expansion phase. *J. Lipid Res.* 2003.** 44: **1652–1659.**

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Cyclo-oxygenase (COX) is the key enzyme required for the conversion of arachidonic acid to prostaglandins. COX catalyses the enzymatic conversion of 20-carbon polyunsaturated fatty acids, such as arachidonic acid, into prostaglandin G2 and H2 (PGH_2), which are subsequently converted into a variety of eicosanoids by specific prostaglandin synthases (1). Two COX isoforms have been described. COX-1 is constitutively expressed in almost all tissues, and its activity depends only on the availability of the substrate. In contrast to COX-1, COX-2 behaves as an immediate early gene subjected to transcriptional regulation. Most of the currently used nonsteroidal anti-inflammatory drugs (NSAIDs) are not specific for any of these COX isoforms and have a number of side effects, mainly linked to their activity on COX-1. Selective COX-2 inhibitors were therefore developed for the management of inflammatory diseases [reviewed in ref. (2)]. Targeting the COX-2 isoform seems to overcome some of the observed side effects, such as the irritation of the gastric mucosa, a problem often encountered with the classical NSAIDs.

Prostaglandins participate in a large number of physiological processes, such as inflammation, immune response, kidney function, bone metabolism, ovulation, and adipocyte differentiation [reviewed in ref. (3)]. Adipocyte differentiation is a complex and staged process (4). Upon reaching confluence, proliferating preadipocytes become growth arrested by contact inhibition. After hormonal induction, those contact-inhibited preadipocytes undergo clonal expansion, characterized by reentry into cell cycle. At this stage, increased cAMP levels elicit the expression of the CCAAT enhancer binding protein transcription factors, C/EBPβ and δ (5, 6), which then induce the expression of peroxisome proliferator-activated receptor γ (PPARγ). In addition, the activity of the E2F family of transcription factors, observed during early phases of adipogenesis, participates in the induction of the expression of PPARγ (7). Both C/EBPs and PPARγ will then trigger the terminal differentiation process. The importance of the early clonal expansion stage is underscored by the observation that either decrease of cAMP levels or inhibition of cell cycle impairs terminal adipocyte differentiation (8).

The role of particular prostaglandins in adipocyte differentiation is suggested to be dependent on the specific stages of this differentiation process. Prostacyclin (PGI2) has been shown to positively influence adipocyte differentiation (8). The stable analog of PGI2, carbacyclin, promotes terminal adipocyte differentiation by eliciting the required increase in cAMP levels as well as the release of

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intracellular Ca^{2+}. Moreover, PGI2 increased the expression of both C/EBPβ and δ in preadipose cells (6). More controversial is the participation of prostaglandin E2 (PGE2). Whereas its antilypolytic effect has been established in differentiated adipocytes (9), PGE2 has been reported to either activate (10) or inhibit (11) early adipogenesis. Finally, prostaglandin J2 (PGJ2) has a positive role in terminal adipocyte differentiation currently thought to be mediated by increasing the activity of PPARγ, a nuclear receptor that also can be activated by a number of synthetic compounds, such as the thiazolidinediones [reviewed in ref. (12)].

By inhibiting prostaglandin synthesis using specific COX-2 inhibitors at different stages of adipogenesis, we demonstrate here that COX-2 plays an important role in early adipocyte differentiation, regulating the entry into cell cycle, whereas its role in terminal differentiation is dispensable.

MATERIALS AND METHODS

Materials

All chemicals, except if stated otherwise, were purchased from Sigma (St. Louis, MO). Antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), except for the anti-bromodeoxyuridine (BrdU) antibody, which was from Sigma. COX-2 inhibitor sc-58236 and COX-1 inhibitor sc-58560 were synthetised by Pharmacia. L165041 was a gift from Merck.

Cell culture and protein extracts

3T3-L1 cells were grown in Dulbecco's modified Eagle medium (DMEM) and 10% fetal calf serum. Cells were differentiated with DMEM, 10% serum, 0.5 mM 3-isobutyl-1methylxanthine, 10 μg/ml insulin, and 1 μM dexamethasone for 2 days. From day 3 on, cells were incubated with DMEM, 10% serum, and 10 μg/ml insulin. Nuclear and whole-cell extracts preparation, Oil red O staining, and quantification are described elsewhere (7).

Immunofluorescence

For all immunofluorescence experiments, cells were grown on coverslips. For BrdU incorporation, cells were incubated for 8 h in the presence of BrdU, and an additional treatment of the cells with 1.5 N HCl for 10 min at 21°C was performed. After fixation and permeabilization with methanol, cells were incubated with the indicated antibodies. Preparations were then incubated with a combination of Texas Red-conjugated anti-mouse IgG and fluorescein isothiocyanate-conjugated anti-rabbit IgG.

Western and Northern blot analyses

SDS-PAGE and electrotransfer were performed as described (13). The membranes were blocked overnight in blocking buffer (20 mM Tris, 100 mM NaCl, 1% Tween-20, and 10% skim milk). Filters were first incubated for 4 h at 21°C with the indicated antibody, and then for 1 h at 21°C with a peroxidase conjugate secondary antibody. The complex was visualized with 4-chloro-1-naphthol. RNA extraction and analysis, as well as aP2 and 36B4 probes, were described previously (14).

Prostaglandins measurement

PGE2 and 15-deoxy Δ PGJ2 levels were determined with the Enzyme Immunoassay Kit exactly as described by the manufacturer (Assay Designs, Inc., Ann Arbor, MI).

RESULTS

COX-2 inhibitors block adipogenesis in 3T3-L1 cells

Western blot analysis of nuclear extracts from differentiating 3T3-L1 preadipocytes demonstrated that COX-2 is absent in quiescent preadipocytes and is robustly induced during the clonal expansion phase of adipogenesis preceding the expression of PPARγ (**Fig. 1A**). E2F1 and cyclin A protein expression were used as markers of the clonal expansion stage (Fig. 1A). The timing of COX-2 expression suggested that it could play a role in this phase of adipocyte differentiation. Therefore, inhibition of COX-2 activity might result in differences in the adipogenic potential of 3T3-L1 cells. 3T3-L1 cells were hence incubated with the specific COX-2 inhibitor sc-58236 for 24 h prior to the induction of adipocyte differentiation with hormonal mix. Consistent with a positive role in adipogenesis, inhibition of COX-2 impaired differentiation in a dose-dependent manner, as evidenced by a decrease in Oil red O staining (Fig. 1B, C) and a decrease in the expression of the adipocyte marker aP2 (Fig. 1D). This inhibitory effect of sc-58236 on adipocyte differentiation correlated with a decrease in the production of PGE2 and 6-keto PGF1α, indicating that the COX-2 enzyme was indeed inactivated (Fig. 1E). Interestingly, when we attempted to measure the effect of the COX-2 inhibitor on the production of the PPARγ agonist 15-deoxy Δ PGJ2, we found barely detectable levels of PGJ2 in any stage of 3T3-L1 differentiation (data not shown). No effect on the production of PGE2 or only a slight effect on the production of 6-keto PGF1α was observed when the specific COX-1 inhibitor, sc-58560, was used in a similar study (Fig. 1E). Furthermore, sc-58560 had no effect on the differentiation of the 3T3-L1 preadipocytes (data not shown).

Because PPARγ is a key player in adipocyte differentiation, we analyzed its expression 48 h after induction of differentiation in the absence or in the presence of 10^{-6} M of the COX-2 inhibitor. Expression of PPARγ was found to be decreased about 3-fold when sc-58236 was added to the differentiation mix (Fig. 1F, G). This result suggested that participation of COX-2 in the differentiation process was not related to PPARγ activation, but was rather affecting any process prior to PPARγ expression.

COX-2 inhibitors repress the clonal expansion phase of adipogenesis

It has been previously reported that COX-2 actively participates in cell cycle progression and proliferation (15, 16). Consequently, inhibition of COX-2 results in cell cycle arrest (15, 16). Because inhibition of cell cycle reentry during clonal expansion inhibits adipocyte differentiation (8), we postulated that inhibition of COX-2 would inhibit cell cycle progression during clonal expansion of adipocyte differentiation. To test this hypothesis, we first analyzed cell cycle progression in proliferating 3T3-L1 preadipocytes. The number of cyclin E (**Fig. 2A**) and BrdU (Fig. 2B) -positive, and thus -proliferating, cells was decreased when the COX-2 inhibitor was used, as measured by immunofluorescence analysis, compared with cells incubated in

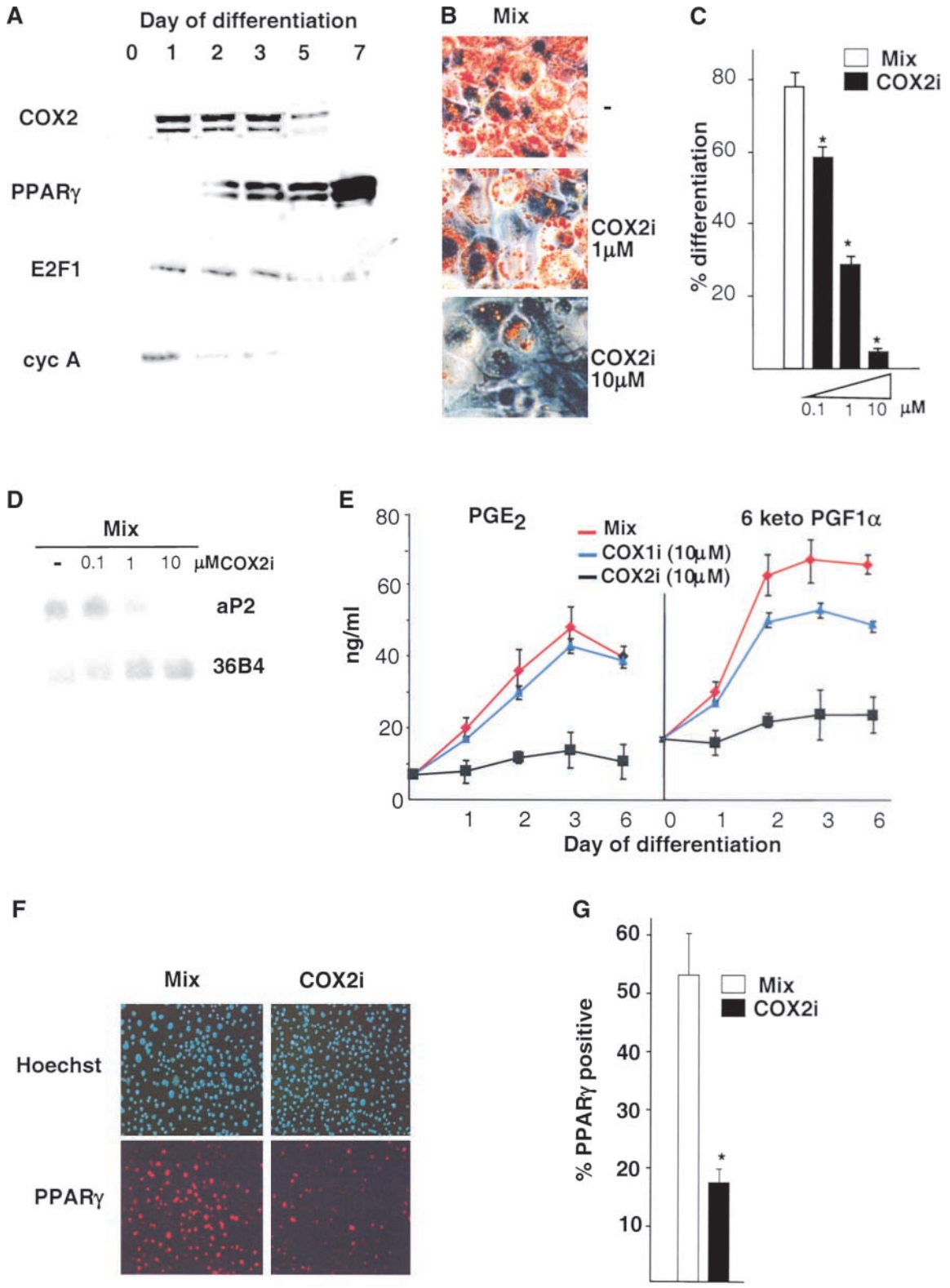

Fig. 1. Cyclo-oxygenase (COX)-2 inactivation inhibits adipocyte differentiation. A: Western blot analysis of whole-cell extracts prepared at different days of adipocyte differentiation of 3T3-L1 cells. The proteins detected with specific antibodies are indicated. B: Oil red O staining comparing lipid accumulation in 3T3-L1 cells induced to differentiate into adipocytes in the absence or presence of 10^{-6} M or 10^{-5} M of the COX-2 inhibitor sc-58236. C: Quantification of the number of differentiated cells by direct counting under light microscopy as a percentage of the total number of cells. At least 500 cells were counted for each individual experiment. Results are the mean of three independent experiments. Statistically significant results ($P < 0.05$) are indicated by an asterisk. D: Northern blot showing the expression of aP2 mRNA in 3T3-L1 cells induced to differentiate for 6 days either under regular conditions as described (Mix), or in the presence of increasing concentrations of COX-2 inhibitor. E: Measurement of prostaglandin E2 and 6 keto PGF1α production at different times of 3T3-L1 adipocyte differentiation in the absence or the presence of COX-2- or COX-1-specific inhibitors. Results are the mean ± SD of three independent measurements. F: Analysis of peroxisome proliferator-activated receptor γ (PPARγ) expression by immunofluorescence in 3T3-L1 cells induced to differentiate for 2 days in the absence (Mix) or the presence of sc-58236 at 10^{-6} M (COX2i). PPARγ-expressing cells are labeled in red. Nuclei were visualized with Hoechst staining. G: Quantification of PPARγ-positive cells in F. At least 500 cells were counted for each condition tested. Statistically significant results ($P < 0.05$) are indicated by an asterisk.

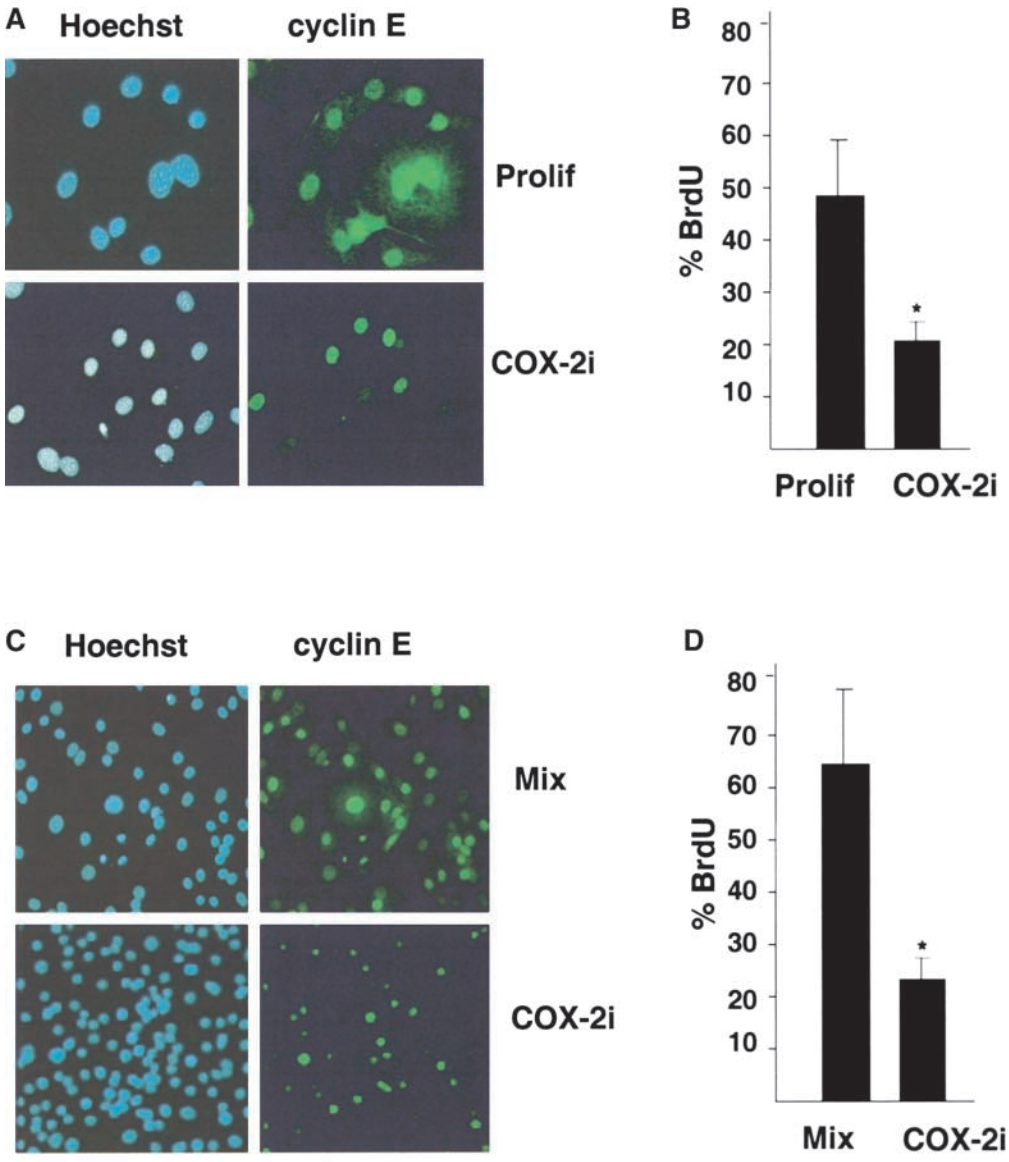

Fig. 2. Inhibition of clonal expansion by COX-2 inhibitors. Analysis of cyclin E expression (A and C) or quantification of bromodeoxyuridine (BrdU) incorporation (B and D) by immunofluorescence in either proliferating 3T3-L1 cells (A and B) or cells induced to differentiate with the hormonal mix (C and D) in the absence or presence of 10^{-6} M of the COX-2 inhibitor. Cyclin E-expressing cells are labeled in green. Nuclei were visualized with Hoechst staining. At least 500 cells were quantified for BrdU incorporation studies. An asterisk indicates statistically significant differences ($P < 0.05$).

the absence of COX-2 inhibitors (Fig. 2A, B). Similarly to proliferating cells, hormonally induced 3T3-L1 preadipocytes incubated in the presence of sc-58236 were prevented from reentering the cell cycle, as indicated by decreased cyclin E expression and BrdU incorporation (Fig. 2C, D). In all cases, no changes in cell density were observed, as determined by cell counting under light microscopy (data not shown). This is somehow surprising, because inhibition of proliferation should result in decreased cell number. However, we have consistently observed increased cell death upon triggering differentiation in the absence of COX inhibitors, which would compensate for the increased cell number as a result of clonal expansion. These results suggested that COX-2 inhibition restrains adipocyte differentiation by repressing the clonal expansion phase.

COX-2 is not required during terminal adipocyte differentiation

In order to more precisely determine the mechanisms involved in the regulation of adipogenesis by COX-2, we added the COX-2 inhibitors either immediately before or 2 days after hormonal induction. Consistent with our previous results, 3T3-L1 cells, which were induced to differentiate in the presence of COX-2 inhibitors, were not able to differentiate (**Fig. 3A**), and consequently did not express

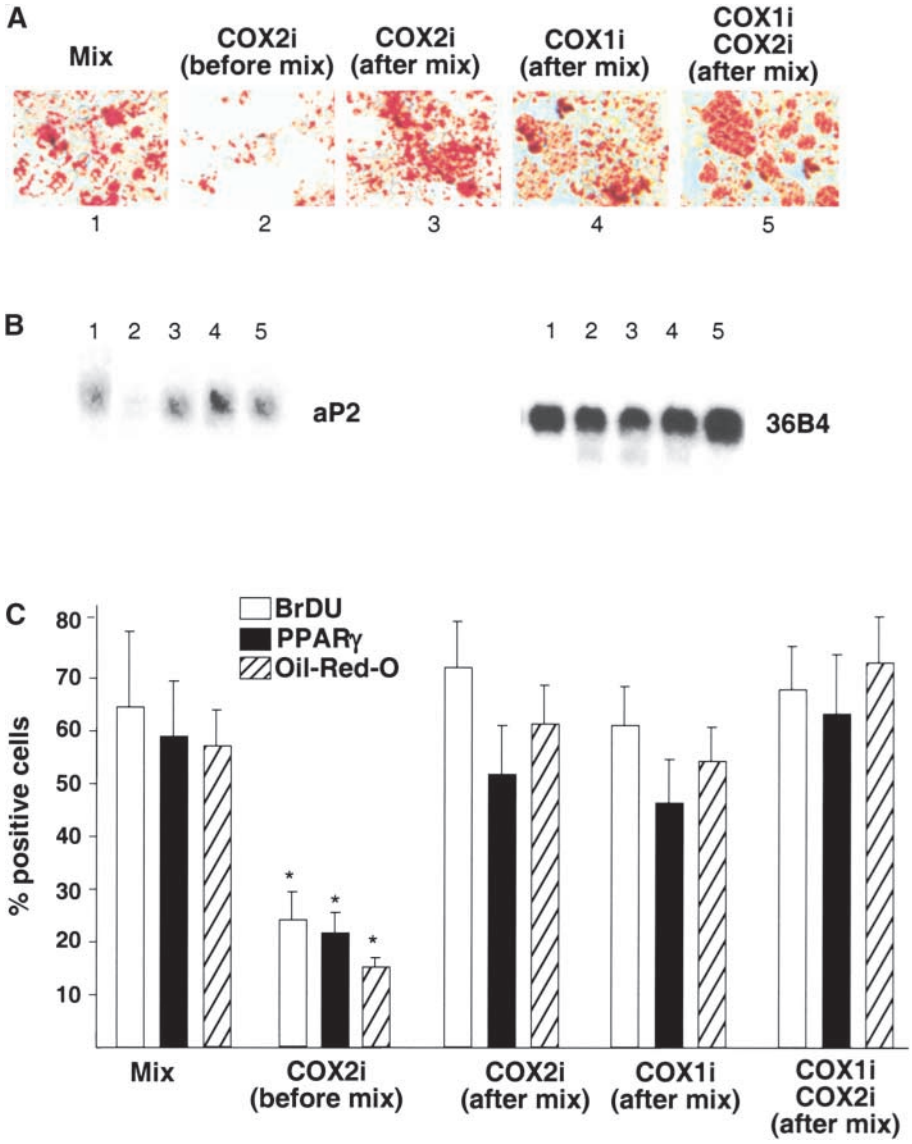

Fig. 3. No effect of COX-2 inhibition in terminal adipocyte differentiation. A: Oil red O staining comparing lipid accumulation in 3T3-L1 cells induced to differentiate into adipocytes in the absence (mix) or presence of 10^{-6} M of sc-58236 (COX2i), added 24 h prior to addition of the differentiation mix (before mix), or in the presence of either sc-58236, sc-58560 (COX1i), or both together, added 48 h after the differentiation mix (after mix). B: Northern blot showing the expression of aP2 mRNA of 3T3-L1 cells used in A. Lane numbers correspond to the conditions used in A. C: Quantification of the number of BrdU-positive, PPARγ-positive, and Oil red O-positive cells analyzed by immunofluorescence or light microscopy under the same conditions tested in A. At least 500 cells were counted in each of three independent experiments. Statistically significant differences ($P < 0.05$) are indicated by an asterisk.

the adipocyte-specific marker aP2 (Fig. 3B). In contrast, adipocyte differentiation and aP2 expression were not affected when COX-2 inhibitors were added after the clonal expansion phase (Fig. 3A, B). Furthermore, neither COX-1 inhibitors nor a combination of COX-1 and COX-2 inhibitors had any effect in the differentiation process when added after the clonal expansion phase (Fig. 3A, B). The number of PPARγ- and BrdU-positive cells was consistent with the observed adipocyte phenotype (Fig. 3C). These results suggest that the role of COX-2 in adipogenesis is restricted to the control of the cell cycle in the early clonal expansion phase.

Mitogenic prostaglandins and PPARβ/δ agonists bypass the block imposed by COX-2 inhibitors on adipogenesis

Inhibition of COX-2 activity results in the depletion of prostaglandins in the cell. Consequently, addition of specific prostaglandins should bypass the block imposed by inhibition of COX-2 activity. To this end, 3T3-L1 cells were induced to differentiate with the regular hormonal mix in the absence or in the presence of the specific COX-2 inhibitor, sc-58236, and specific prostaglandins. Cells were then allowed to differentiate for 7 days. It is important to note that cells were cultured in the presence of prosta-

glandins only for 2 days, the time required for the clonal expansion phase. In this way, further effects of prostaglandins not related to clonal expansion were minimized. When immunofluorescence analysis was performed to quantify BrdU incorporation at the second day of differentiation, cells incubated without sc-58236 showed a substantial increase of BrdU-positive cells, which is consistent with the reentry of the cells into cell cycle (**Fig. 4A, B**). In contrast, a clear reduction of BrdU-positive cells was observed when COX-2 was inhibited (Fig. 4A, B). Addition of PGE2 and 6-keto PGF1α resulted in an increase of BrdU-positive cells similar to that observed when cells were cultured in the absence of COX-2 inhibitors (Fig. 4A, B). PGA2 and PGJ2, however, were unable to reverse the reduction in BrdU-positive cells upon COX-2 inhibition (Fig. 4A, B). Strikingly, only cells that reentered cell cycle, and thus underwent clonal expansion, were able to express PPARγ, incorporate lipids, and consequently differentiate into adipocytes at day 7 (Fig. 4A, B). These results suggest that proliferative prostaglandins, such as PGE2 and the PGI2 derivative, 6-keto PGF1α, contribute to the clonal expansion phase of adipogenesis. 6-Keto PGF1α is a PGI2 metabolite, which is a PPARβ/δ activator, suggesting that the observed effects of this prostaglandin could be mediated at least in part by PPARβ/δ. Because expression of PPARβ/δ was not affected in COX-2-inhibited preadipocytes (data not shown), we evaluated the effects of PPARβ/δ activation in these cells. Interestingly, addition of the synthetic PPARβ/δ agonist L165041 promoted cell cycle reentry and was also able to bypass the block imposed by the COX-2 inhibitor in adipocyte differentiation (Fig. 4A, B).

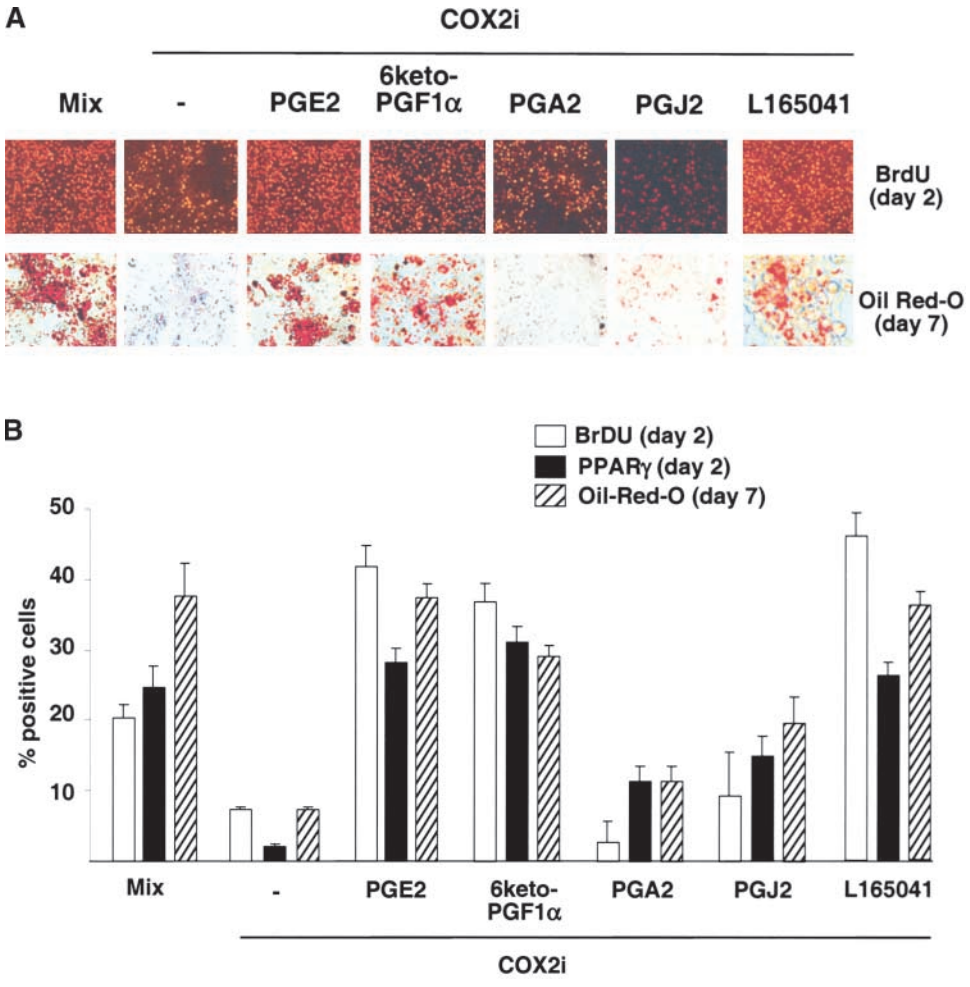

Fig. 4. Proliferative prostaglandins and PPARβ/δ agonists bypass the block of COX-2 inhibitors in adipogenesis. A: Analysis of both BrdU incorporation and Oil red O staining in postconfluent 3T3-L1 cells stimulated with differentiation medium (mix) for 2 days in the absence or presence of 10^{-6} M COX-2 inhibitor with or without the addition of the indicated prostaglandins or the PPARβ/δ agonist ligand L165041. Prostaglandins were only present in the differentiation medium for 2 days at a final concentration of 10^{-6} M. At this time, cells were fixed and immunofluorescence assay was performed to detect BrdU incorporation (red labeling). Nuclei were stained with the Hoechst reagent (blue staining). For Oil red O staining, cells were allowed to differentiate for 5 additional days. B: Quantification of the number of BrdU-positive (2 days), PPARγ-positive (3 days), or Oil red O-stained cells (7 days) analyzed by immunofluorescence under the conditions tested in A.

DISCUSSION

Arachidonic acid, through the action of COX, is converted into prostaglandins, which are major regulators of cell growth, differentiation, and homeostasis [reviewed in ref. (3)]. In this study, we demonstrate that inhibition of COX-2 attenuates adipocyte differentiation, which is consistent with the previously reported adipogenic potential of some prostaglandins (17, 18). Our first hypothesis regarding participation of COX-2 in adipogenesis was that it facilitates the synthesis of prostaglandins, ligands of PPARγ. Notably, PGJ2 has been demonstrated to promote adipocyte differentiation through activation of PPARγ (19, 20). Therefore, inactivation of COX-2 could result in the depletion of PGJ2 in the cell. Consequently, PPARγ would remain inactive, and cells would fail to differentiate. However, a more detailed analysis of the effects of COX-2 inhibition demonstrated that adipocyte differentiation was attenuated before PPARγ was expressed. This suggested that other PPARγ-independent mechanisms were causing the block in adipocyte differentiation imposed by selective COX-2 inhibition. An argument supporting this hypothesis was that inhibition of prostaglandin synthesis during terminal adipocyte differentiation, when the clonal expansion phase was completed, did not affect the adipogenic potential. Although PGJ2, as a PPARγ ligand, has adipogenic potential, the fact that inhibition of COX-2 activity at later stages of adipogenesis had no major effect on terminal differentiation raises the question also of the importance of the participation of this prostaglandin in the adipocyte differentiation process. Supporting this idea is the fact that the PPARγ ligand 15-deoxy Δ PGJ2 remains undetectable in 3T3-L1 cells independent of the differentiation stage (data not shown). The previous report that the PGJ2 precursor, PGD2, is only present at very low levels in epididymal fat is consistent with a minor role of this prostaglandin, as well as its downstream products in adipose tissue biology (21).

Having excluded an eventual role of COX-2 as a supplier of PPARγ ligands in terminal differentiation, we turned our attention to the eventual effects of COX-2 and prostaglandins in the early phases of adipocyte differentiation. Reentry of growth-arrested preadipocytes into cell cycle in response to hormonal stimulation is a prerequisite for adipocyte differentiation, because the inhibition of DNA synthesis at this stage results in the attenuation of differentiation (8). Our data clearly suggest that COX-2 inhibition abrogates clonal expansion, because most of the cells did not reenter the cell cycle, as measured by BrdU incorporation and cyclin E expression. Consistent with this, only prostaglandins, which exhibited a proliferative potential, were able to overcome the block in adipogenesis imposed by the COX-2 inhibitor. Of particular physiological relevance is the fact that both PGE2 and the metabolite of PGI2, 6-keto PGF1α (Fig. 4), which are the two major prostaglandins produced by adipose tissue and adipose precursor cells (9), could trigger reentry into cell cycle and therefore adipogenesis. PGI2 is a positive effector of adipocyte differentiation, exerting its effect through elevating cAMP levels in preadipocytes, activating the CREB/ATF pathway (22). This facilitates the proliferative response of the cell and therefore promotes clonal expansion (22).

Interestingly, PGI2 could be a positive effector of adipogenesis through regulating PPARβ/δ activity. Indeed, it has been previously shown that PGI2 activates this nuclear receptor (23). This is furthermore consistent with the reported positive role of PPARβ/δ during the clonal expansion phase of adipogenesis (24, 25) and the fact that in the present study the synthetic PPARβ/δ agonist L165041 overcame the effect of COX-2 inhibition on cell proliferation. Furthermore, significant amounts of 6-keto PGF1α are produced during adipocyte differentiation (Fig. 1E). These effects of PPARβ/δ activation on clonal expansion in adipocyte differentiation are furthermore in line with the proliferative effects of PPARβ/δ activation in other systems, such as colon (26). In the colon, PPARβ/δ was shown to be a target gene for the β-catenin/Tcf-4 transcription complex, which is formed when the adenomatous polyposis coli tumor suppressor protein is mutated. In view of the coexpression of PPARβ/δ and COX-2 (23), it was proposed that PPARβ/δ can mediate the protumorigenic effects of prostaglandins in the colon, whereas NSAIDs were suggested to inhibit tumorigenesis because they inhibit PPARβ/δ activity (26). The lack of tumorigenicity of PPARβ/$\delta^{-/-}$ human colorectal cells in nude mice supported a protumorigenic role of PPARβ/δ (27).

PGE2 plays also a clear role in differentiated adipocytes inhibiting lipolysis, whereas its role in preadipocytes is less studied. We show here that PGE2 stimulates proliferation of 3T3-L1 cells induced to differentiate, suggesting that PGE2 positively influences adipocyte differentiation through triggering the clonal expansion phase (Fig. 4). Participation of PGE2 in proliferative processes, such as liver regeneration after partial hepatectomy, has been previously documented (28). It has also been demonstrated that this effect is mediated through the PGE2 receptor EP1, which activates the cAMP pathway (29). Interestingly, preadipocytes express a higher level of EP1 receptor compared with mature adipocytes (30). We also tested the effect of other prostaglandins in adipogenesis. Consistent with previous studies, PGF2α inhibited the whole adipocyte differentiation process (11, 31), whereas no effect was observed for PGA2, PGD2, PGJ2, or TXB2 (Fig. 4 and data not shown).

In contrast to our results, a recent study documented a negative role of COX-1 and COX-2 in adipocyte differentiation (32). In this study, inhibition of COX activity resulted in a slight increase in adipogenesis. However, it is not clear in this study whether COX inhibitors were added before or after clonal expansion. Furthermore, COX-1 and -2 activity was not measured in this study at each particular time of the differentiation process. It is therefore not possible to know whether clonal expansion was abrogated.

Obesity is a major health problem in developed countries and is characterized by an increase in size of the adi-

pocytes, as a result of lipid accumulation, and an increase in the number of adipocytes, as a result of accelerated adipocyte differentiation. COX-2-selective inhibitors are currently being used for the treatment of inflammatory diseases and are under clinical trials for the treatment of cancer (33). Interestingly, obesity has recently been suggested to be a low-grade systemic inflammatory disease (34). Therefore, inhibition of the inflammatory response may also attenuate the formation of fat. We present here evidence to support the idea that modulation of the activity of the COX-2 enzyme might open up new perspectives in the control of adipogenesis and consequently for the treatment of obesity and related metabolic diseases.

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