Arachidonic acid and prostacyclin signaling promote adipose tissue development: a human health concern?

Florence Massiera,* Perla Saint-Marc,* Josiane Seydoux,† Takahiko Murata,§ Takuya Kobayashi,§ Shuh Narumiya,§ Philippe Guesnet, Ez-Zoubir Amri,* Raymond Negrel,* and Gérard Ailhaud[1,*]**

Institut de Recherche Signalisation,* Biologie du Développement et Cancer, Centre de Biochimie (UMR6543CNRS), UNSA, Faculté des Sciences, Parc Valrose, 06108 Nice cedex 2, France; Centre Médical Universitaire,† Département de Physiologie, 1 rue Michel Servet, 1211 Genève 4, Switzerland; Department of Pharmacology,§ Kyoto University, Faculty of Medicine, Yoshida, Sakyo-ku, Kyoto 606-8315, Japan; and Laboratoire de Nutrition et Sécurité Alimentaire,** INRA, 78352 Jouy-en-Josas, France

Abstract **High fat intake is associated with fat mass gain through fatty acid activation of peroxisome proliferator-activated receptors δ and γ, which promote adipogenesis. We show herein that, compared to a combination of specific agonists to both receptors or to saturated, monounsaturated, and ω-3 polyunsaturated fatty acids, arachidonic acid (C20:4, ω-6) promoted substantially the differentiation of clonal preadipocytes. This effect was blocked by cyclooxygenase inhibitors and mimicked by carbacyclin, suggesting a role for the prostacyclin receptor and activation of the cyclic AMP-dependent pathways that regulate the expression of the CCAAT enhancer binding proteins β and δ implicated in adipogenesis. During the pregnancy-lactation period, mother mice were fed either a high-fat diet rich in linoleic acid, a precursor of arachidonic acid (LO diet), or the same isocaloric diet enriched in linoleic acid and α-linolenic acid (LO/LL diet). Body weight from weaning onwards, fat mass, epididymal fat pad weight, and adipocyte size at 8 weeks of age were higher with LO diet than with LO/LL diet. In contrast, prostacyclin receptor-deficient mice fed either diet were similar in this respect, indicating that the prostacyclin signaling contributes to adipose tissue development. These results raise the issue of the high content of linoleic acid of *i*) ingested lipids during pregnancy and lactation, and *ii*) formula milk and infant foods in relation to the epidemic of childhood obesity.**—Massiera, F., P. Saint-Marc, J. Seydoux, T. Murata, T. Kobayashi, S. Narumiya, P. Guesnet, E-Z. Amri, R. Negrel, and G, Ailhaud. **Arachidonic acid and prostacyclin signaling promote adipose tissue development: a human health concern?** ***J. Lipid Res.*** **2003.** 44: **271–279.**

Supplementary key words prostacyclin receptor-deficient mice • adipogenesis • pregnancy-lactation • childhood obesity

Obesity is associated with metabolic disorders such as dyslipidemia, diabetes, and hypertension, and fat mass excess in severe obesities is typically due to an increase in adipocyte size and number. The formation of adipocytes is a critical event, as mature adipocytes do not divide in vivo and do not undergo significant turnover under physiological conditions. The capacity for proliferation of precursor cells and their differentiation into adipocytes is highest at early age and decrease thereafter in humans and rodents. A limited number of hormones can affect the adipose tissue mass and possibly its distribution (1). High dietary fat intake is now recognized to be associated with a gain of fat mass in animals and humans at all ages (2–5). However, the lack of evidence of a general increase in energy intake as fat among youths, despite a striking increase in the prevalence of obesity in industrial and developing countries, may be due in part to decreased physical activity and nonexercise activity thermogenesis (6), but also to the composition of food intake in early life. The long-term relationship between the fatty acid composition of dietary fats and the development of adipose tissue in humans is difficult to assess in contrast to animals. When mother rats were fed a high-fat diet rich in linoleic acid (C18:2, ω-6) or saturated fatty acids, suckling pups at 17 days of age exhibited hyperplasia or hypertrophy of white adipose tissue, respectively (7). Moreover, fish oil rich in eicosapentaenoic acid (C20:5, ω-3, EPA) and docosahexaenoic acid (C22:6, ω-3, DHA) prevents obesity in rats (8, 9), as well as feeding rats after weaning with dietary fats rich in α-linolenic acid (C18:3, ω-3), the precursor of EPA and DHA, prevents excessive growth of adipose tissue (10).

The mechanisms underlying the differential adipogenic effect of ω-6 versus ω-3 polyunsaturated fatty acids

Manuscript received 29 August 2002 and in revised form 28 October 2002.

Published, JLR Papers in Press, November 4, 2002.
DOI 10.1194/jlr.M200346-JLR200

Abbreviations: ALBP (aP2), adipocyte lipid binding protein; PKA, protein kinase A.

[1] To whom correspondence should be addressed.
e-mail: ailhaud@unice.fr

suggest differences between fatty acids and/or fatty acid metabolites in promoting differentiation of adipose precursor cells into adipocytes. In vitro, at the preadipocyte stage, a member of the peroxisome proliferator-activated receptor (PPAR) family, i.e., PPARδ, and two members of the CCAAT-enhancer binding protein family, i.e., C/EBPβ and C/EBPδ, act concomitantly to upregulate the subsequent and critical expression of PPARγ leading to adipogenesis (11–15). Natural long-chain fatty acids act in preadipocytes as adipogenic hormones, participate as transcriptional regulators of the expression of various lipid-related genes, and promote adipogenesis (16). These effects implicate PPARs that bind long-chain fatty acids and fatty acid metabolites (17). Among fatty acids, arachidonic acid (C20:4, ω-6, ARA), a precursor of prostaglandin I_2 (prostacyclin), synthesized and released from preadipocytes, has been identified as one of the main adipogenic components of serum. Arachidonic acid induces a rapid cAMP production. Both this effect and its long-term adipogenic effect are impaired by cyclooxygenase inhibitors such as aspirin and indomethacin (18). Consistent with an autocrine-paracrine mechanism via released prostacyclin, antibodies directed against this prostanoid and added externally decrease by half the adipogenic effect of arachidonic acid (19). Also consistent with a role of prostacyclin acting as a ligand at the cell surface, it has been shown that *i*) prostacyclin and its stable analog carbacyclin mimic the effects of arachidonic acid (20) and also promote adipogenesis of clonal mouse preadipocytes and primary preadipocytes from rat and human (21), and *ii*) prostacyclin binding to its cell surface receptor (IP-R) activates in preadipocytes the protein kinase A (PKA) pathway (21) and upregulates the early expression of the C/EBPβ and C/EBPδ (22). Circumstantial evidence favors the possibility that prostacyclin also binds like carbacyclin to PPARδ (23), and this has been recently supported by studies on stromal cells surrounding the implanting blastocysts (24).

In vivo, invalidation of C/EBPβ and C/EBPδ genes impairs severely but does not abolish adipose tissue formation (25), whereas invalidation of PPARδ gene leads to a controversial disproportionate decrease in fat mass (26, 27). This suggests that prostacyclin signaling, arising from arachidonic metabolism, may play a more important adipogenic role through C/EBPβ and C/EBPδ than through PPARδ in up-regulating PPARγ expression. In order to estimate the relative importance of the two regulatory pathways, we have taken advantage of the recent availability of specific PPAR agonists and the generation of prostacyclin receptor-null (*ip-r*$^{-/-}$) mice (28).

Our results with wild-type and *ip-r*$^{-/-}$ mice show that polyunsaturated fatty acids of the ω-6 and ω-3 series are not equipotent in promoting adipogenesis both in vitro and in vivo, and that arachidonic acid and prostacyclin signaling favor this process. In infants, given the relative enrichment of various foods in linoleic acid as precursor of arachidonic acid, its excessive consumption at a time where adipose tissue is in a dynamic phase of its development may favor childhood obesity.

MATERIALS AND METHODS

Mice

The *ip-r*-null mice were established by gene targeting and backcrossed with C57/BL6J mice for at least 10 generations (28), then *ip-r*$^{-/-}$ males and *ip-r*$^{-/-}$ females were bred to generate further generations. Both *ip-r*$^{-/-}$ and C57/BL6J control mice were maintained on a light/dark cycle with light from 6 AM to 6 PM at 25°C. The female mice designated to be mothers were fed either a standard diet which consisted (by energy) of 7% fat, 66% carbohydrates, and 27% proteins, or a high-fat diet containing 15% corn oil (LO diet) or a mixture of 10% corn oil and 5% perilla oil (LO/LL diet). Both high-fat diets consisted (by energy) of 40% fat, 35% carbohydrates, and 25% proteins, and were supplemented with 0.04% vitamin C and 0.02% vitamin E (UAR, Carbon Blanc, France). Corn oil contained, expressed in g/100 g of total fatty acids, 13% saturated, 27% monounsaturated, 59% ω-6 polyunsaturated, and 1% ω-3 polyunsaturated fatty acids. The mixture of corn oil and perilla oil contained 10.9% saturated, 22.9% monounsaturated, 44.3% ω-6 polyunsaturated, and 21.9% ω-3 polyunsaturated fatty acids. At 8 weeks of age, female mice fed the same diet since weaning were bred to male mice and maintained throughout mating, pregnancy, and lactation on the same diet. At 18 days of age, male pups were weaned onto the same diets that their mothers had consumed and maintained thereafter. Food intake, body weight, body composition, and cellularity measurements of epididymal fat pad were performed as described previously (29). All experimental animal protocols were performed in accordance with the recommendations of the French Accreditation of Laboratory Animal Care.

Fibroblast culture

Embryos from C57 BL/6J wild-type and *ip-r*$^{-/-}$ mice at 14.5 day postcoitus were used to prepare fibroblasts after removing head, heart, and legs. Mouse embryo fibroblasts were then differentiated into adipocytes as previously described (22).

Preadipocyte culture

Stock cultures of Ob1771 cells were maintained in Dulbecco's modified Eagle's medium (Gibco, Cergy-Pontoise, France) supplemented with biotin, pantothenate, antibiotics, and 8% (v/v) fetal bovine serum as previously described (18). Experiments were performed after growth and differentiation of confluent cells in serum-free medium as previously described (18). Fatty acids, prostaglandins (Cayman Chemicals, Montluçon, France), GW2433, and BRL49653 were dissolved in ethanol and added at a 1:100 dilution into culture media. Ethanol concentration, which did not exceed 1%, had no effect on either adipose conversion or cyclic AMP production. Oil-Red O staining was performed as described previously (18).

Biochemical assays

Glycerol-3-phosphate dehydrogenase assays were carried out in duplicate at day 7 after confluence (18). Intracellular cyclic AMP was determined with a commercial kit by radioimmunoassay, according to the manufacturer's instructions (Amersham). Before assays, cyclic AMP was extracted with 1.2 ml of ice-cold ethanol-5 mM-EDTA (2:1, v/v). After scraping the cell monolayer and centrifugation, 1 ml of the supernatant was dried in a Speed-Vac evaporator. Duplicate samples were solubilized before assays in 150 μl of 50 mM Tris-HCl buffer, pH 7.5, containing 4 mM EDTA and assayed at least in duplicate.

Statistical analysis

Statistical comparisons were performed on absolute values by Fisher's PLSD using the STATVIEW software package.

RESULTS

ω-6 ARA but not ω-3 ARA promotes adipogenesis

We performed experiments in confluent preadipocytes exposed for 7 days to serum-free medium in the absence (**Fig. 1A**) or the presence of various effectors. Differentiation was enhanced in cells exposed to the naturally abundant ω-6 ARA (Fig. 1B) compared with ω-3 ARA, which is only present at trace amounts in dietary fat sources (Fig. 1C). Differentiation in the presence of ω-6 ARA was severely impaired when aspirin was included as a cyclooxygenase inhibitor (Fig. 1D). Another polyunsaturated fatty acid of the ω-3 series, DHA, behaved similarly to ω-3 ARA (Fig. 1E). In agreement with our previous observations (20), carbacyclin, a stable prostacyclin analog that binds to the cell surface prostacyclin receptor IP-R, was highly adipogenic (Fig. 1F), exhibiting greater activity than GW2433, a specific PPARδ agonist (17) (Fig. 1G), or a combination of GW2433 and BRL49663, a specific PPARγ agonist (30) (Fig. 1H). In order to delineate at which step ω-6 ARA was active in the differentiation process, we first studied adipose conversion in the presence of maximally effective concentrations of PPARδ and/or PPARγ agonist using glycerol-3-phosphate dehydrogenase activity, an adipocyte indicator.

The sequential addition of PPAR agonists was based upon several considerations. *i*) Early markers are expressed in PPARγ$^{-/-}$ embryonic stem cells that can be committed into the adipose lineage, i.e., lipoprotein lipase and PPARδ but not the adipocyte lipid binding protein (ALBP or aP2) (31); *ii*) PPARδ is expressed at near maximal levels in early confluent Ob1771 cells, in contrast to PPARγ, which is highly expressed later during adipogenesis (32); and *iii*) activation of PPARδ by activators-ligands is required to upregulate the expression of PPARγ (33, 34).

Consistent with these observations, addition of the PPARδ agonist from day 0 to day 3, followed by its removal and addition of the PPARγ agonist from day 3 to day 7, proved to be optimal. Adipogenesis was similar to that observed in the presence of both agonists between day 0 and day 7. In contrast, discontinuous or continuous exposure to either agonist for 7 days was less efficient in promoting adipogenesis (**Table 1**, part A). Of note, when the PPARγ agonist was present from day 3 to day 7, the adipogenic potency of ω-6 ARA added during the first 3 days was 3-fold higher than that of the PPARδ agonist and this effect was severely reduced after cyclooxygenase inhibition (Table 1, part A). In contrast to ω-6 ARA and carbacyclin, the adipogenic potencies of ω-3 ARA and PPARδ agonist were similar. Interestingly, in the presence of the PPARδ agonist between day 0 and day 3, ω-6 and ω-3 ARA exhibited an adipogenic potency similar to that of the PPARγ agonist (Table 1, part A). Altogether, these results show that the substantial effect of ω-6 ARA takes place at early step(s) of the differentiation process, in accordance with the fact that we did not observe prostacyclin synthesis and response through IP-R in differentiating, PPARγ-expressing cells (21).

In order to gain further insights into the role of IP-R, adipogenesis of mouse embryo fibroblasts from wild-type and *ip-r*$^{-/-}$ mice (22) was compared upon stimulation by a combination of BMY45778, a specific agonist of IP-R like carbacyclin but unable to activate PPARδ (35), and the PPARγ agonist. Adipogenesis was decreased 2-fold in *ip-r*$^{-/-}$ fibroblasts compared with that of wild-type fibroblasts while exposure to a combination of BMY45778 and PPARγ agonist did not enhance adipogenesis above that observed with the PPARγ agonist alone (data not shown).

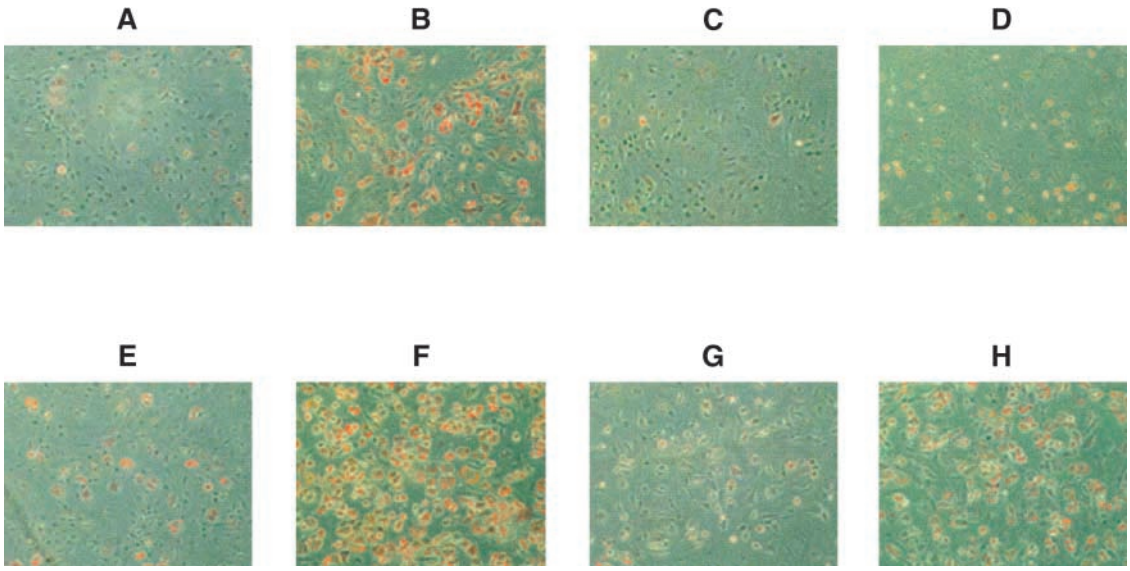

Fig. 1. Photomicrographs of confluent Ob1771 preadipocytes maintained for 7 days and stained with Oil Red-O in serum-free medium (A) or in serum-free medium supplemented with 10 μM ω-6 arachidonic acid (ARA) (B), 10 μM ω-3 ARA (C), 10 μM ω-6 ARA and 100 μM aspirin (D), 10 μM docosahexaenoic acid (DHA) (E), 1 μM carbacyclin (F), 1 μM GW2433 as peroxisome proliferator-activated receptor (PPAR)δ agonist (G), 1 μM GW2433, and 0.5 μM of BRL49653 as PPARγ agonist (H). Magnification, 600-fold.

TABLE 1. Comparative stimulation of adipogenesis by PPAR agonists and long-chain fatty acids

Addition from Day 0 to Day 3	Addition from Day 3 to Day 7	GPDH Activity (Fold Increase)
Part A		
None	None	1.0[a]
None	γ agonist	2.4 ± 0.6
δ agonist	None	3.6 ± 0.8[b]
δ agonist	γ agonist	5.7 ± 1.6[c]
δ agonist	δ agonist	2.1 ± 1.0
γ agonist	γ agonist	5.2 ± 1.0[c]
δ + γ agonists	δ + γ agonists	6.6 ± 2.1[c]
5 μM ω-6 ARA	γ agonist	9.3 ± 2.1[c]
10 μM ω-6 ARA	γ agonist	17.5 ± 6.2[c]
5 μM ω-6 ARA + 100 μM aspirin	γ agonist	4.4 ± 1.4[b]
5 μM ω-3 ARA	γ agonist	2.6 ± 0.7[b]
10 μM ω-3 ARA	γ agonist	5.5 ± 1.2[c]
5 μM ω-3 ARA + 100 μM aspirin	γ agonist	1.5 ± 0.3
δ agonist	5 μM ω−6 ARA	4.9 ± 2.3[b]
δ agonist	5 μM ω−3 ARA	5.8 ± 1.2[c]
Carbacyclin	γ agonist	17.3 ± 3.0[c]
Part B		
5 μM Palmitic acid	γ agonist	1.9 ± 0.6
10 μM Palmitic acid	γ agonist	2.3 ± 0.5[b]
5 μM Palmitoleic acid	γ agonist	3.0 ± 0.6[b]
10 μM Palmitoleic acid	γ agonist	3.5 ± 0.8[b]
5 μM Oleic acid	γ agonist	2.7 ± 0.6[b]
10 μM Oleic acid	γ agonist	2.2 ± 0.4[b]
5 μM EPA[d]	γ agonist	3.5 ± 0.9[b]
5 μM DHA	γ agonist	4.1 ± 1.2
10 μM DHA	γ agonist	5.5 ± 0.9[b]

ARA, arachidonic acid; δ agonist, GW2433 (1 μM); γ agonist, BRL49653 (0.5 μM); carbacyclin (0.2 μM). Values are expressed as mean ± SEM of experiments performed on 3–10 independent series of cells.

[a] 30 ± 3 mU/mg of protein.

[b] $P < 0.05$ versus untreated cells.

[c] $P < 0.01$ versus untreated cells.

[d] 10 μM EPA proved to be cytotoxic on a long-term basis.

As no expression of C/EBPβ and C/EBPδ was observed in *ip-r*$^{-/-}$ mouse embryo fibroblasts in response to carbacyclin (22), it appears that the remarkable adipogenic potency of carbacyclin was likely due to its dual role, first as a ligand of IP-R and activation of the PKA pathway leading in turn to C/EBPβ and C/EBPδ expression and up-regulation of PPARγ expression (36), and second as a ligand of PPARδ.

Only ω-6 ARA promotes extensive adipogenesis and cyclic AMP production

The potency of various long-chain fatty acids to stimulate early events of differentiation was next examined in preadipocytes exposed subsequently to the PPARγ agonist (Table 1, part B). A saturated fatty acid (palmitate) or monounsaturated fatty acids (palmitoleate and oleate) was poorly adipogenic compared with ω-6 ARA. Furthermore, two ω-3 polyunsaturated fatty acids, EPA and DHA, were also poorly adipogenic. The higher adipogenic activity of ω-6 ARA compared with other fatty acids cannot be ascribed to its higher affinity for PPARδ, as the latter binds arachidonic acid, saturated, monounsaturated, and ω-3 polyunsaturated fatty acids with similar affinity (17). As ω-6 ARA was unique among natural fatty acids in promoting extensive adipocyte differentiation, we compared cyclic AMP production after a very short exposure of preadipocytes to concentrations of the various long-chain fatty acids (**Table 2**). ω-6 ARA increased cyclic AMP production by 15-fold in 5 min in confluent preadipocytes, and this effect was abolished by indomethacin, another cyclooxygenase inhibitor. Carbacyclin increased cyclic AMP production by 13-fold and, as anticipated, indomethacin had no effect. Compared with control cells, PKA activity was increased 3- and 4-fold in the presence of ω-6 ARA (10 μM) and carbacyclin (1 μM), respectively (data not shown). ω-3 ARA, EPA, DHA, palmitic acid, palmitoleic acid, oleic acid, PPARδ agonist, or PPARγ agonist had no effect on cyclic AMP production. In order to investigate why ω-3 polyunsaturated fatty acids were not potent adipogenic agents, we attempted to inhibit PPARδ and/or PPARγ activity in the presence of ω-3 ARA. Adipogenesis was unaffected compared with that observed in the presence of PPAR agonists only, excluding PPARs as possible targets of ω-3 ARA (data not shown). Thus, we investigated in preadipocytes the possible effect on cyclic AMP production of ω-3 polyunsaturated fatty acids that have been reported to inhibit the cyclic AMP-dependent PKA (37). In the presence of ω-6 ARA (5 μM), addition of ω-3 ARA, EPA, or DHA (10 μM each) inhibited cyclic AMP production by 51%, 91%, and 9% respectively, whereas, in the presence of ω-6 ARA (10 μM), a 33% inhibition of this production was observed by addition of EPA (10 μM) (not shown). These results suggest that ω-3 polyunsaturated fatty acids inhibit in preadipocytes the cyclic AMP-signaling pathways triggered by arachidonic acid at level(s) upstream of PKA.

In vivo ω-6 polyunsaturated fatty acids are more adipogenic than ω-3 polyunsaturated fatty acids

To address whether the fatty acid composition of high-fat diets promotes adipose tissue development, we carried

TABLE 2. Comparative cyclic AMP production by arachidonic acid and various effectors of adipogenesis

Addition	cAMP Production[a]
	pmol/5 min per well
None	1.0
ω-6 ARA	16.2
ω-6 ARA + indomethacin	1.5
ω-3 ARA	1.8
ω-3 ARA + indomethacin	1.6
δ agonist	1.8
γ agonist	1.2
Carbacyclin	13.9
Carbacyclin + indomethacin	11.0
Palmitic acid	1.9
Oleic acid	1.4
EPA	3.1
DHA	0.2

[a] Values are expressed as mean of duplicate values representative of experiments performed on three independent series of cells. Fatty acids and indomethacin (added 15 min prior to fatty acid) were present at 10 μM; δ agonist, GW 2433 (1 μM); γ agonist, BRL 49653 (0.5 μM); carbacyclin (0.2 μM).

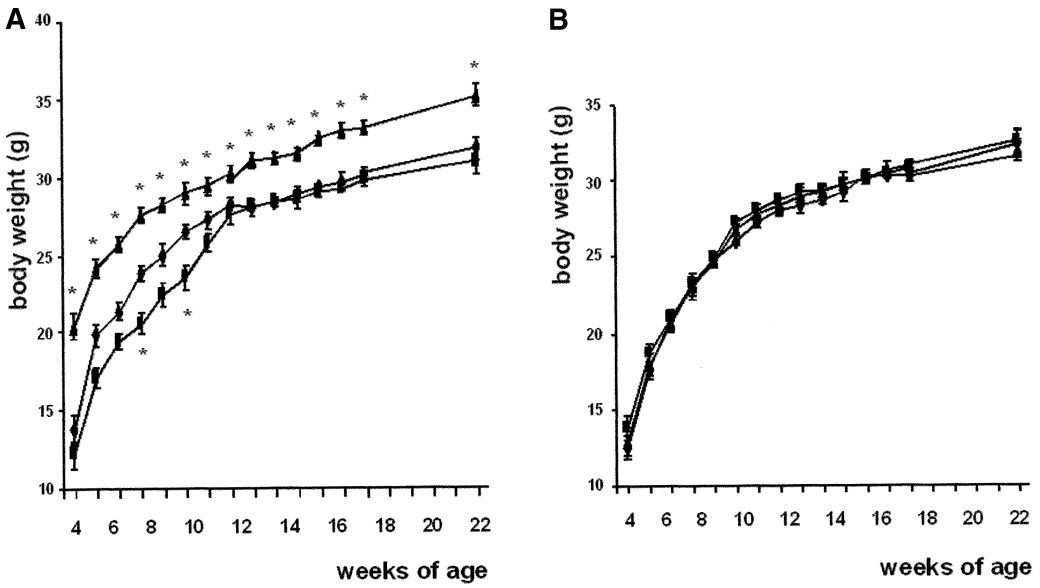

Fig. 2. Body weight of wild-type (A) and *ip-r*$^{-/-}$ male mice (B). During the pregnancy-lactation period, mother mice were fed a standard diet (diamond), a corn oil-enriched diet (triangle), or a mixture of corn oil and perilla oil-enriched diet (square). From weaning onward, male pups were maintained on the same diet than their mothers had consumed. (n = 20), *$P < 0.05$ versus wild-type mice fed a standard diet.

out nutritional studies in wild-type and *ip-r*$^{-/-}$ mice. We chose a corn oil-supplemented diet rich in linoleic acid, a long-known essential fatty acid precursor of arachidonic acid (LO diet) compared with an isocaloric diet enriched with a mixture of corn oil and perilla oil rich in α-linolenic acid, another essential fatty acid precursor of EPA and DHA (LO/LL diet). The female mice designated to be mothers were fed diets containing either LO diet or LO/LL diet from 4 weeks of age. Four weeks later, these mice were bred to male mice and remained on the same diet. Pups were kept with their mothers until weaning and then maintained on the same respective diets.

From weaning to 22 weeks of age, body weight of wild-type mice fed LO diet was higher than that of animals fed LO/LL diet and this difference persisted to a large extent at the adult age (**Fig. 2**) despite a similar food intake (**Table 3**). Strikingly, the body weight of *ip-r*$^{-/-}$ mice on either type of diets was similar. As the amount of food intake was also similar despite differences in caloric intake, this suggests increased thermogenesis in *ip-r*$^{-/-}$ mice fed a high-fat diet. Moreover, body weight of *ip-r*$^{-/-}$ mice was indistinguishable when fed LO diet or LO/LL diet, demonstrating the critical role of ω-6 polyunsaturated fatty acids in body weight gain during pregnancy and/or the suckling period. No difference was observed in body length between wild-type and *ip-r*$^{-/-}$ mice fed either type of diets. To evaluate adiposity, we measured total fat mass and epididymal fat pad weight of wild-type and *ip-r*$^{-/-}$ mice at 8 weeks of age (Table 3). Fat mass of wild-type mice fed standard diet or LO/LL diet was identical, whereas that of animals fed LO diet was increased. In contrast, fat mass was identical in *ip-r*$^{-/-}$ mice fed either diet. Consistent with these observations, epididymal fat pad weight of wild-type mice was significantly higher in mice fed LO diet than in mice fed LO/LL diet or standard diet. Again, there was no significant difference in the epididymal fat pad weights of *ip-r*$^{-/-}$ mice fed either type of diets. Adipocyte size of epididymal fat pad was increased 1.9-fold in wild-type mice fed LO diet compared with the two other diets, and this was accompanied by a decrease of ad-

TABLE 3. Food intake, fat mass, and epididymal fat pad weight in 8-week-old wild type and *ip-r*$^{-/-}$ mice fed either a standard diet (Chow) or a high-fat diet enriched with linoleic acid (LO) or enriched with linoleic acid and α-linolenic acids (LO/LL)

	Wild-Type			*ip-r*$^{-/-}$		
	Chow Diet	LO Diet	LO/LL Diet	Chow Diet	LO Diet	LO/LL Diet
Food intake (g/day) (n = 4)	4.7 ± 0.5	4.5 ± 0.6	4.7 ± 0.6	4.8 ± 0.9	4.4 ± 0.4	4.8 ± 0.6
Total fat mass (g) (n = 4)	2.3 ± 0.1	2.7 ± 0.1[a]	2.3 ± 0.1	1.8 ± 0.1	1.6 ± 0.1	1.8 ± 0.1
Epididymal fat pads (mg) (n = 6)	307.6 ± 7.5	396 ± 15.1[b]	282 ± 18.2	214.5 ± 7.2	209.8 ± 3.7	227.4 ± 9.8

[a] $P < 0.08$ versus Chow-fed.
[b] $P < 0.01$ versus Chow-fed.

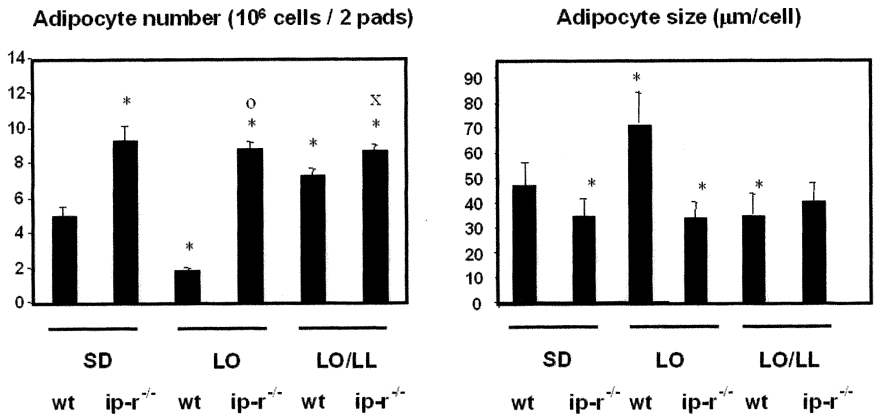

Fig. 3. Adipocyte number and size in epididymal fat pad of 8-week-old male wild-type and *ip-r*$^{-/-}$ mice (n = 3 in each group) fed after weaning to a standard diet (SD), LO diet, or LO/LL diet. Values are expressed as mean ± SEM; * $P < 0.05$ versus wild-type fed a standard diet. O, $P < 0.05$ versus wild-type fed LO diet; X, $P < 0.01$ versus wild-type fed LO/LL diet.

ipocyte number (**Fig. 3**). Compared with wild-type mice, adipocyte size was decreased and adipocyte number was increased in *ip-r*$^{-/-}$ mice fed standard diet, yet there was no difference in adipocyte size and number of the epididymal fat pad of *ip-r*$^{-/-}$ mice fed either diet (Fig. 3). Surprisingly, when wild-type mother mice were fed a standard diet and pups were fed after weaning a LO or LO/LL diet, body weight of animals fed LO diet was not significantly

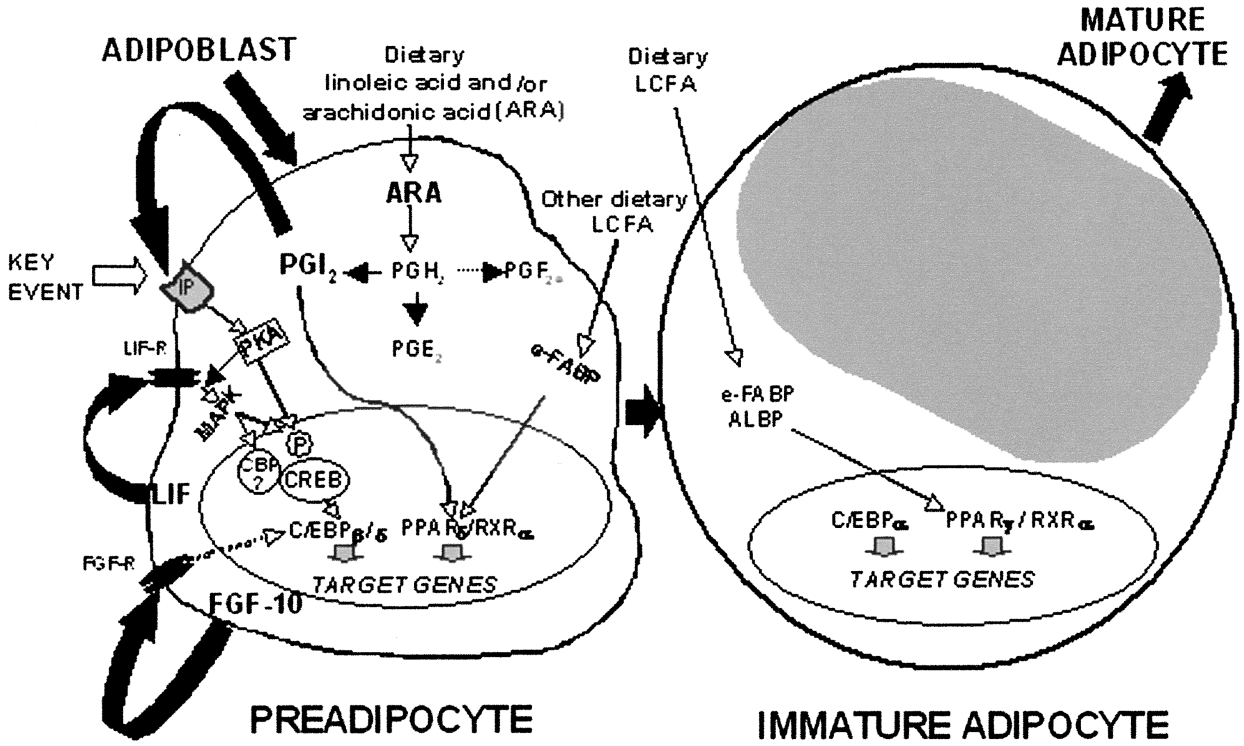

Fig. 4. Redundant pathways and dietary long chain-fatty acids (LCFA) implicated in adipogenesis. This scheme assumes that three ligand/cell surface receptor systems concur to upregulate the expression of C/EBPβ and C/EBPδ, i.e., prostacyclin/IP-R (22, 39), leukemia inhibitory factor (LIF) receptor (22, 39), and fibroblast growth factor (FGF)-10 receptor (48), and to promote adipogenesis. Dietary linoleic acid and/or arachidonic acid favor in preadipocytes prostacyclin synthesis and release. Thus, arachidonic acid via prostacyclin triggers a key event, plays a unique role in activating the protein kinase A pathway by means of IP-R, and enhances the differentiation process. Other dietary LCFA behave as mere activators/ligands of PPARδ and subsequently of PPARγ. Upon terminal differentiation, leukemia inhibitory factor and fibroblast growth factor-10 are no longer produced. Production of prostacyclin and other prostaglandins ceases and is accompanied by reduced expression and loss of functional IP (21, 49, 50). Therefore, in adipocytes, PPARγ and possibly PPARδ then act as the molecular sensors of all dietary LCFA at a time where endogenous LCFA synthesis (not shown) becomes significant (51). Epidermal (keratinocyte) fatty acid binding protein in preadipocytes and adipocytes and adipocyte lipid binding protein in adipocytes are assumed to bind and transport LCFA (51, 52).

higher than that of those fed LO/LL diet, emphasizing the importance of linoleic acid-enriched diet during the pregnancy-lactation period.

DISCUSSION

The development of adipose tissue depends on the existence of redundant pathways that ultimately upregulate the expression of PPARγ. This expression relies predominantly on the expression of C/EBPβ and C/EBPδ (36) and disruption of both genes severely impairs the formation of adipose tissue (25). We had identified cues that trigger the expression of C/EBPβ and C/EBPδ in preadipocytes, i.e., leukemia inhibitory factor and its cognate cell surface receptor activating the ERK pathway (38) and the prostacyclin/IP-R system activating the PKA pathway (22). Our present results show that polyunsaturated fatty acids of the ω-6 series are more adipogenic both in vitro and in vivo compared with their ω-3 counterparts. On one hand, ω-6 ARA is unique via prostacyclin in activating the PKA pathway. On the other hand, ω-3 polyunsaturated fatty acids do not affect this pathway as well as prostaglandin I_3, a product synthesized from EPA (not shown). **Figure 4** summarizes our views on redundant pathways and on the role of arachidonic acid and other long-chain fatty acids in promoting adipogenesis.

In light of our results from in vitro studies reported herein, a high-fat diet rich in linoleic acid could stimulate arachidonic acid formation and, through prostacyclin synthesis, activate cyclic AMP-dependent signaling pathways in preadipocytes. This would favor the formation of mature adipocytes whose lipid filling is then increased to cope with the high exogenous levels of fatty acids. In contrast, in wild-type mice fed a high-fat diet containing a mixture of linoleic and α-linolenic acids, the lowered production of cyclic AMP may limit the formation of adipocytes, leading to hyperplasia to accommodate the fatty acid supply. This alteration of cyclic AMP production could be due to a decreased arachidonic acid synthesis from linoleic acid through inhibition of Δ6 desaturase activity by α-linolenic acid and its metabolites (39). In *ip-r*$^{-/-}$ mice, no activation of the PKA pathway occurs through the prostacyclin receptor, and the adipogenic effect of ω-6 and ω-3 polyunsaturated fatty acids is similar. Despite prostacyclin synthesis in *ip-r*$^{-/-}$ mice, no difference in adipose tissue mass was observed between mice fed LO or LO/LL diet, suggesting that the activation of PPARδ by prostacyclin, if any, had no significant impact on the overall differentiation process. Our finding that inclusion of α-linolenic acid in an isocaloric diet rich in linoleic acid prevents the enhancement of fat mass is consistent with our in vitro observations. Varying the proportion of these essential fatty acids thus should alter the proportion of arachidonic acid versus EPA and DHA (39) and could lead to changes in the pattern of adipose tissue development which occurs during pregnancy and the suckling period.

Remarkably, although no difference in the litter size was observed in wild-type mice fed either diet, pups from mother mice fed LO diet were, at weaning, 50% heavier than those from mice fed LO/LL diet. This raises an important issue in humans, as adipose tissue is being formed during the third trimester of gestation and sensitive periods of its development take place before 1 year of age (1). In this respect, the overweight prevalence among US children of 6 to 11 months of age increased in boys from 4.0% in 1976 through 1980 to 7.5% in 1988 though 1994, and in girls from 6.2% to 10.8% during the same periods of time (40). It is of interest to observe that the content of linoleic acid of mature breast milk of US women, a reflection of their fat intake, has increased from 5% to 17% between 1945 and 1995 ($r = 0.85$, $P < 0.001$, $n = 29$). Furthermore, despite the fact that the ratio of linoleic acid to α-linolenic acid in mature breast milk of US and European women is similar, it is also interesting to note that the ratio of arachidonic acid to DHA is 1.8-fold higher in the milk of US women due to its low DHA content (41–43). It has been reported that breast feeding may help decrease the prevalence of overweight and obesity in childhood (44,45). Although the enhanced fatness was attributed to the greater energy intake of formula-fed infants (46), the fatty acid composition should be considered, as the percentage of linoleic acid is increased by approximately 50% in infant formula compared with breast milk (47). We suggest that, at a very early age where energy expenditure appears rather similar between individuals, large amounts of linoleic acid consumed during pregnancy, suckling period, and early infancy are important determinants of physiological events implicated at a time when adipose tissue is in a dynamic phase of its development, and that could lead to childhood obesity.

This study was supported by grants from Institut Français pour la Nutrition (F.M.) and CERIN (G.A.), by a special grant from the Bristol Myers Squibb Foundation (G.A.), and by a grant from the Swiss National Science Foundation (no. 31-57-129.99 to J.S.). Special thanks are due to G. Oillaux for skillfull secretarial assistance, to C. Vernochet for drawings, and to Dr. R. Arkowitz for critical review of the manuscript. GW2433 and BRL49653 were kind gifts of S. Michel and U. Reichert (Galderma, Sophia Antipolis, France), and BMY 45778 was a kind gift of N. Meanwell (Bristol-Myers Squibb). The authors gratefully acknowledge the generous gift of perilla oil from Dr. Kajiwava (Ajimoto Co., Inc., Suzuki-cho, Japan).

REFERENCES

1. Ailhaud, G., and H. Hauner. 1997. Development of white adipose tissue. *In* Handbook of Obesity. G. Bray, C. Bouchard, and P. T. James, editors. M. Dekker Inc., New York, NY. 359–378.
2. Troiano, R. P., R. R. Briefel, D. M. Carrol, and K. Bialostosky. 2000. Energy and fat intakes of children and adolescents in the United States: data from the National Health and Nutrition examination surveys. *Am. J. Clin. Nutr.* **72:** 1343S–1353S.
3. Oscai, L. B., N. M. Brown, and W. C. Miller. 1984. Effect of dietary fat on food intake, growth and body composition in rats. *Growth.* **48:** 415–424.
4. Romieu, I., W. C. Willett, M. J. Stampfer, G. A. Colditz, L. Sampson, B. Rosner, C. H. Hennckens, and F. E. Speizer. 1988. Energy

intake and other determinants of relative weight. *Am. J. Clin. Nutr.* **74:** 406–412.
5. Nguyen, T., D. E. Larson, R. K. Johnson, and M. I. Goran. 1996. Fat intake and adiposity in children of lean and obese parents. *Am. J. Clin. Nutr.* **63:** 507–513.
6. Levine, J. A., N. L. Eberhardt, and M. D. Jensen. 1999. Role of nonexercise activity thermogenesis in resistance to fat gain in humans. *Science.* **283:** 212–214.
7. Clearly, M. P., F. C. Philips, and A. A. Morton. 1999. Genotype and diet effects in lean and obese Zucker rats fed either safflower or coconut oil diets. *Proc. Soc. Exp. Biol. Med.* **220:** 153–161.
8. Parrish, C. C., D. A. Pathy, and A. Angel. 1990. Dietary fish oils limit adipose tissue hypertrophy in rats. *Metabolism.* **39:** 217–219.
9. Raclot, T., R. Groscolas, D. Langin, and P. Ferré. 1997. Site-specific regulation of gene expression by n-3 polyunsaturated fatty acids in rat white adipose tissues. *J. Lipid Res.* **38:** 1963–1972.
10. Okuno, M., K. Kajiwara, S. Imai, T. Kobayashi, N. Honma, T. Maki, K. Suruga, T. Goda, S. Takase, Y. Muto, and H. Moriwaki. 1997. Perilla oil prevents the excessive growth of visceral adipose tissue in rats by down-regulating adipocyte differentiation. *J. Nutr.* **127:** 1752–1757.
11. Barak, Y., M. C. Nelson, E. S. Ong, Y. Z. Jones, P. Ruiz-Lozano, K. R. Chien, A. Kader, and R. M. Evans. 1999. PPARγ is required for placental, cardiac, and adipose tissue development. *Mol. Cell.* **4:** 585–595.
12. Kubota, N., Y. Terauchi, H. Miki, H. Tamemoto, T. Yamauchi, K. Komeda, S. Satoh, R. Nakano, C. Ishii, T. Sugiyama, K. Eto, Y. Tsubamoto, A. Okuno, K. Murakami, H. Sekihara, G. Hasegawa, M. Naito, Y. Toyoshima, S. Tanaka, K. Shiota, T. Kitamura, T. Fujita, O. Ezaki, S. Aizawa, R. Nagai, K. Tobe, S. Kimura, and T. Kadowaki. 1999. PPARγ mediates high-fat diet-induced adipocyte hypertrophy and insulin resistance. *Mol. Cell.* **4:** 597–609.
13. Rosen, E. D., P. Sarraf, A. E. Troy, G. Bradwin, K. Moore, D. S. Milstone, B. M. Spiegelman, and R. M. Mortensen. 1999. PPARγ is required for the differentiation of adipose tissue *in vivo* and *in vitro*. *Cell.* **4:** 611–617.
14. Ren, D., T. N. Collingwood, E. J. Rebar, A. P. Wolffe, and H. S. Camp. 2002. PPARγ knockdown by engineered transcription factors: exogenous PPARγ2 but not PPARγ1 reactivates adipogenesis. *Genes Dev.* **16:** 27–32.
15. Rosen, E. D., C. H. Hsu, X. Wang, S. Sakai, M. W. Freeman, F. J. Gonzalez, and B. M. Spiegelman. 2002. C/EBPα induces adipogenesis through PPARγ: a unified pathway. *Genes Dev.* **16:** 22–26.
16. Amri, E., G. Ailhaud, and P. Grimaldi. 1994. Fatty acids as signal transducing molecules: involvement in the differentiation of preadipose to adipose cells. *J. Lipid Res.* **35:** 930–937.
17. Xu, H. E., M. H. Lambert, V. G. Montana, D. J. Parks, S. G. Blanchard, P. J. Brown, D. D. Sternbach, J. M. Lehmann, G. B. Wisely, T. M. Willson, S. A. Kliewer, M. V. Milburn. 1999. Molecular recognition of fatty acids by peroxisome proliferator-activated receptors. *Mol. Cell.* **3:** 397–403.
18. Gaillard, D., R. Négrel, M. Lagarde, and G. Ailhaud. 1989. Requirement and role of arachidonic acid in the differentiation of preadipose cells. *Biochem. J.* **257:** 389–397.
19. Catalioto, R. M., D. Gaillard, J. Maclouf, G. Ailhaud, and R. Négrel. 1991. Autocrine control of adipose cell differentiation by prostacyclin and $PGF_{2\alpha}$. *Biochim. Biophys. Acta.* **1091:** 364–369.
20. Négrel, R., D. Gaillard, and G. Ailhaud. 1989. Prostacyclin as a potent effector of adipose cell differentiation. *Biochem. J.* **257:** 399–405.
21. Vassaux, G., D. Gaillard, G. Ailhaud, and R. Négrel. 1992. Prostacyclin is a specific effector of adipose cell differentiation: its dual role as a cAMP- and Ca^{2+}-elevating agent. *J. Biol. Chem.* **267:** 11092–11097.
22. Belmonte, N., B. W. Phillips, F. Massiéra, P. Villageois, B. Wdziekonski, P. Saint-Marc, J. Nichols, J. Aubert, K. Saeki, A. Yuo, S. Narumiya, G. Ailhaud, C. Dani. 2001. Activation of extracellular signal-regulated kinases and CREB/ATF-1 mediate the expression of C/EBPβ and C/EBPδ in preadipocytes. *Mol. Endocrinol.* **15:** 2037–2049.
23. Forman, B. M., J. Chen, and R. M. Evans. 1997. Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors α and δ. *Proc. Natl. Acad. Sci. USA.* **94:** 4312–4317.
24. Lim, H., R. A. Gupta, W. Ma, B. C. Paria, D. E. Moller, J. D. Morrow, R. N. DuBois, J. M. Trzaskos, and S. K. Dey. 1999. Cyclo-oxygenase-2-derived prostacyclin mediates embryo implantation in the mouse via PPARδ. *Genes Dev.* **13:** 1561–1574.
25. Tanaka, T., N. Yoshida, T. Kishimoto, and S. Akira. 1997. Defective adipocyte differentiation in mice lacking the C/EBPβ and/or C/EBPδ gene. *EMBO J.* **16:** 7432–7443.
26. Peters, J. M., S. S. T. Lee, W. Li, J. M. Ward, O. Gavrilova, C. Everett, M. L. Reitman, L. D. Hudson, and F. J. Gonzalez. 2000. Growth, adipose, brain and skin alterations resulting from targeted disruption of the mouse peroxisome proliferator-activated receptor β (δ). *Mol. Cell. Biol.* **20:** 5119–5128.
27. Barak, Y., D. Liao, W. He, F. S. Ong, M. C. Nelson, J. M. Olefsky, R. Boland, and R. M. Evans. 2002. Effects of peroxisome proliferator-activated receptor delta on placentation, adiposity and colorectal cancer. *Proc. Natl. Acad. Sci. USA.* **99:** 303–308.
28. Murata, T., F. Ushikubi, T. Matsuoka, M. Hirata, A. Yamasaki, Y. Sugimoto, A. Ichikawa, Y. Aze, T. Tanaka, N. Yoshida, S. Oh-ishi, and S. Narumiya. 1997. Altered pain perception and inflammatory response in mice lacking prostacyclin receptor. *Nature.* **388:** 678–682.
29. Massiéra, F., J. Seydoux, A. Geloen, A. Quignard-Boulangé, S. Turban, P. Saint-Marc, A. Fukamizu, R. Négrel, G. Ailhaud, and M. Teboul. 2001. Angiotensinogen-deficient mice exhibit impairment of diet-induced weight gain with alteration in adipose tissue development and increased locomotor activity. *Endocrinology.* **142:** 5220–5225.
30. Lehmann, J. M., L. B. Moore, T. A. Smith-Oliver, W. O. Wilkison, T. M. Willson, and S. A. Kliewer. 1995. An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor γ (PPARγ). *J. Biol. Chem.* **270:** 12953–12956.
31. Vernochet, C., D. S. Milstone, C. Iehlé, N. Belmonte, B. Phillips, B. Wdziekonski, P. Villageois, E. Z. Amri, P. E. O'Donnell, R. M. Mortensen, G. Ailhaud, and C. Dani. 2002. PPARγ-dependent and PPARγ-independent effects on the development of adipose cells from embryonic stem cells. *FEBS Lett.* **510:** 94–98.
32. Amri, E. Z., F. Bonino, G. Ailhaud, N. Abumrad, and P. A. Grimaldi. 1995. Cloning of a protein that mediates transcriptional effects of fatty acids in preadipocytes. Homology to peroxisome proliferator-activated receptors. *J. Biol. Chem.* **270:** 2367–2371.
33. Bastié, C., S. Luquet, D. Holst, C. Jehl-Pietri, and P. Grimaldi. 1999. Expression of peroxisome proliferator-activated receptor PPARδ promotes induction of PPARγ and adipocyte differentiation in 3T3C2 fibroblasts. *J. Biol. Chem.* **274:** 21920–21925.
34. Hansen, J. B., H. Zhang, T. H. Rasmussen, R. K. Petersen, E. N. Flindt, and K. Kristiansen. 2001. Peroxisome proliferator-activated receptor δ (PPARδ)-mediated regulation of preadipocyte proliferation and gene expression is dependent on cAMP signaling. *J. Biol. Chem.* **276:** 3175–3182.
35. Aubert, J., P. Saint-Marc, N. Belmonte, C. Dani, R. Négrel, and G. Ailhaud. 2000. Prostacyclin IP receptor up-regulates the early expression of C/EBPβ and C/EBPδ in preadipose cells. *Mol. Cell. Endocrinol.* **160:** 149–156.
36. Wu, Z., N. L. Bucher, and S. R. Farmer. 1996. Induction of peroxisome proliferator-activated receptor gamma during the conversion of 3T3 fibroblasts into adipocytes is mediated by C/EBPbeta, C/EBPdelta and glucocorticoids. *Mol. Cell. Biol.* **16:** 4128–4136.
37. Mirnikjoo, B., S. E. Brown, H. F. Seung Kim, L. B. Marangell, J. D. Sweatt, and E. J. Weeber. 2001. Protein kinase inhibition by ω-3 fatty acids. *J. Biol. Chem.* **276:** 10888–10896.
38. Aubert, J., S. Dessolin, N. Belmonte, M. Li, F. R. McKenzie, L. Staccini, P. Villageois, B. Barhanin, A. Vernallis, A. G. Smith, G. Ailhaud, and C. Dani. 1999. Leukemia inhibitory factor and its receptor promote adipocyte differentiation via the mitogen-activated protein kinase cascade. *J. Biol. Chem.* **274:** 24965–24972.
39. Raz, A., N. Kamin-Belsky, F. Przedecki, and M. G. Obukowicz. 1997. Fish oil inhibits Δ6 desaturase activity *in vivo*: utility in a dietary paradigm to obtain mice depleted of arachidonic acid. *J. Nutr. Biochem.* **5:** 558–565.
40. Ogden, C. L., R. P. Troiano, R. R. Briefel, R. J. Kuczmarski, K. M. Flegal, and C. L. Johnson. 1997. Prevalence of overweight among preschool children in the United States, 1971 through 1994. *Pediatrics.* **99:** E1–E7.
41. Jensen, R. G. 1989. Fatty acids and related compounds. *In* The Lipids of Human Milk. R. G. Jensen, editor. CRC Press, Inc. Boca Raton, Florida. 93–151.
42. Jensen, R. G. 1997. The lipids in human milk. *Prog. Lipid Res.* **35:** 53–92.
43. Jensen, R. G. 1999. Lipids in human milk. *Lipids.* **34:** 1243–1271.
44. Von Kries, R., B. Koletzko, T. Sauerwald, E. von Mutius, D. Barnert, V. Grunert, and H. von Voss. 1999. Breast feeding and obesity: cross sectional study. *BMJ.* **319:** 147–150.

45. Gillman, M. W., S. L. Rifas-Shiman, C. A. Camargo, Jr., C. S. Berkey, A. L. Frazier, H. R. H. Rockett, A. E. Field, and G. A. Colditz. 2001. Risk of overweight among adolescents who were breastfed as infants. *JAMA*. **285:** 2461–2467.
46. Stunkard, A. J., R. I. Berkowitz, V. A. Stallings, and D. A. Schoeller. 1999. Energy intake, not energy output, is a determinant of body size in infants. *Am. J. Clin. Nutr.* **69:** 524–530.
47. Guesnet, P., P. Pugo-Gunsam, C. Maurage, M. Pinault, B. Giraudeau, J-M. Alessandri, G. Durand, J-M. Antoine, and C. Couet. 1999. Blood lipid concentrations of docosahexaenoic and arachidonic acids at birth determine their relative postnal changes in term infants fed breast milk or formula. *Am. J. Clin. Nutr.* **70:** 292–298.
48. Sakaue, H., M. Konishi, W. Ogawa, T. Asaki, T. Mori, M. Yamasaki, M. Takata, H. Ueno, S. Kato, M. Kasuga, and N. Itoh. 2002. Requirement of fibroblast growth factor 10 in development of white adipose tissue. *Genes Dev.* **16:** 908–912.
49. Börglum, J., B. Richelsen, C. Darimont, S. B. Pedersen, and R. Négrel. 1997. Expression of the two isoforms of prostaglandin endoperoxide synthase (PGHS-1 and PGHS-2) during adipose cell differentiation. *Mol. Cell. Endocrinol.* **131:** 67–77.
50. Vassaux, G., D. Gaillard, C. Darimont, G. Ailhaud, and R. Négrel. 1992. Differential response of preadipocytes and adipocytes to PGI_2 and PGE_2: physiological implications. *Endocrinology.* **131:** 2393–2398.
51. Ibrahimi, A., L. Teboul, D. Gaillard, E. Amri, G. Ailhaud, P. Young, M. Cawthorne, and P. Grimaldi. 1994. Evidence for common mechanism of action for fatty acids and thiazolidinedione antidiabetic agents on gene expression in preadipose cells. *Mol. Pharmacol.* **46:** 1070–1076.
52. Shaughnessy, S., E. R. Smith, S. Kodukula, J. Storch, and S. K. Fried. 2000. Adipocyte metabolism in adipocyte fatty acid binding protein knockout (aP2−/−) mice after short-term high-fat feeding: functional compensation by keratinocyte fatty acid binding protein. *Diabetes.* **49:** 904–911.