Bisphenol A in combination with insulin can accelerate the conversion of 3T3-L1 fibroblasts to adipocytes

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Abstract The confluent cultures of 3T3-L1 fibroblasts were treated with or without bisphenol A (BPA) for 2 days and subsequently treated with insulin (INS) alone for 9 days. When BPA was absent during the first 2-day treatment period, the cultures contained 1.6 μg/μg DNA of triacylglycerol (TG), 202 mU/mg DNA of lipoprotein lipase (LPL) activity, and 462 nmol/min/mg DNA of glycerol-3-phosphate dehydrogenase (GPDH) activity. The presence of BPA during the same period caused a 150% increase in the TG content, a 60% increase in the LPL activity, and a 500% increase in the GPDH activity. Thus, BPA by itself can trigger 3T3-L1 fibroblasts to differentiate into adipocytes. Next, the confluent cultures were treated with BPA for 2 days and subsequently treated with a combination of INS and BPA for 9 days. The simultaneous presence of BPA with INS caused a 370% increase in the TG content, a 200% increase in the LPL activity, and a 225% increase in the GPDH activity compared with the cultures treated with INS alone. The amount of [^{3}H]thymidine incorporated into DNA was lower in the cultures treated with INS in the presence of BPA than in those treated with INS alone, indicating that BPA has an anti-proliferative activity on 3T3-L1 cells. Taken together, our results indicate that BPA in combination with INS can accelerate the adipocyte conversion.—Masuno, H., T. Kidani, K. Sekiya, K. Sakayama, T. Shiosaka, H. Yamamoto, and K. Honda. **Bisphenol A in combination with insulin can accelerate the conversion of 3T3-L1 fibroblasts to adipocytes. *J. Lipid Res.* 2002.** 43: **676–684.**

Supplementary key words environmental endocrine disrupting chemical • lipoprotein lipase • glycerol-3-phosphate dehydrogenase • triacylglycerol accumulation • 3T3-L1 adipocytes

Bisphenol A (BPA) is an environmental endocrine disrupting chemical that affects reproduction in wildlife (1, 2). BPA is a monomer of polycarbonate plastics and a constituent of epoxy and polystyrene resins, which are used in the food packing industry. BPA contained in lacquer coatings of food cans is found as a contaminant not only in the liquid of the preserved foods, but also in the water autoclaved in the cans (3). This chemical is also released from polycarbonate flasks during autoclaving (4). Moreover, it has been reported that significant amounts of BPA were detected in the saliva of dental patients treated with fissure sealants (5).

BPA has been shown to mimic the actions of estrogens (6). Estrogens are known to affect lipid metabolism in adipose tissue. In the rat, the administration of estrogens induced a depletion of the triacylglycerol (TG) stores (7). This estrogen-induced depletion of TG has been explained by a decreased activity of adipose tissue lipoprotein lipase (LPL) (7, 8), an enzyme which plays a key role in TG accumulation in adipocytes. Thus, estrogens regulate body weight and adiposity by acting on the processes controlling the TG mass. However, there have been very few reports on the effect of BPA on lipid metabolism in adipose tissue. A recent report showed that BPA diglycidyl ether (BPADGE), but not BPA, decreased TG accumulation in cultured adipocytes (9).

Green et al. (10–12) have clonally isolated a cell line of mouse fibroblasts (3T3-L1 cells) that can differentiate into adipocytes. This cell line offers an excellent model system for the study of differentiation processes (10–18). A variety of reagents that trigger these fibroblasts to differentiate into adipocytes have been identified. These triggers include insulin (INS) (12, 13, 15), dexamethasone (DEX) (14), 1-methyl-3-isobutylxanthine (MIX) (13, 15), prostaglandin F2α (PG F2α) (12, 13, 15), prolactin (13), and sodium butyrate (16). The most efficient means to trigger the differentiation is to treat the confluent cul-

Abbreviations: BPA, bisphenol A; BPADA, bisphenol A diacetate; BPABCF, bisphenol A bis(chloroformate); BPADGE, bisphenol A diglycidyl ether; DEX, dexamethasone; GPDH, glycerol-3-phosphate dehydrogenase; INS, insulin; LPL, lipoprotein lipase; MIX, 3-isobutyl-1-methylxanthine; TG, triacylglycerol.

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tures of 3T3-L1 fibroblasts with a combination of INS, DEX, and MIX for 2 days (14, 17, 18).

In the present study, we determined whether the environmental endocrine disrupting chemicals, especially BPA, affect the conversion of 3T3-L1 fibroblasts to adipocytes.

MATERIALS AND METHODS

Materials

BPA, bisphenol A diacetate (BPADA), bisphenol A bis(chloroformate) (BPABCF), and bisphenol A diglycidyl ether (BPADGE) were from Tokyo Kasei Co., Tokyo, Japan. Bovine insulin, dexamethasone, and 3-isobutyl-1-methylxanthine were from Sigma. [^{3}H]thymidine and enhanced chemifluorescence (ECF) substrate were from Amersham Pharmacia Biotech. Mouse monoclonal antibody to proliferating cell nuclear antigen (PCNA) was from DAKO Japan Co., Kyoto, Japan. Alkaline phosphatase-conjugated goat anti-mouse IgG was from ICN Phrmaceuticals, Inc. Polyvinylidene difluoride (PVDF) membrane was from Bio-Rad Laboratories. A kit for TG was from Wako Pure Chemicals Co., Osaka, Japan. All other chemicals were highest quality commercially available.

Cell culture treatment

3T3-L1 fibroblasts were grown to confluence in a culture medium containing 10% (v/v) FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B in DMEM, on a 60-mm plate. Confluent cells were cultured in the absence or presence of 20 μg/ml BPA for 2 days. The medium was then replaced with a culture medium containing either 5 μg/ml INS alone or a combination of 5 μg/ml INS and 20 μg/ml BPA and changed every 2 days. Nine days later, the cells were harvested in 1.2 ml of 50 mM NH_4Cl/NH_4OH buffer (pH 8.2) containing 20 μg/ml heparin and 2% (w/v) BSA and sonicated briefly at 0°C for preparation of acetone/ether powders and measurements of DNA and TG.

Cultures that were cultured for 2 days in the presence of a combination of 10 μg/ml INS, 1 μM DEX, and 0.5 mM MIX after confluence and then treated for 9 days with 5 μg/ml INS alone were used as the positive control cultures.

Lipid staining of cells

The cultures were fixed with 10% (v/v) formalin in Dulbecco's PBS, and then stained with Oil Red O as described by Kuri-Harcuch and Green (19). To quantitate the percentage of lipid-positive cells in the cultures, at least 1,000 cells/60-mm plate were counted.

Assay of LPL activity

The cell-associated LPL activity was measured in aqueous extracts of acetone/ether powders of cells (20). The extract was made by adding the powder to ice-cold 50 mM NH_4Cl/NH_4OH buffer (pH 8.2) containing 20 μg/ml heparin, letting the mixture stand at 0°C for 1 h, sonicating briefly at 0°C, centrifuging, and decanting the supernatant for assay.

A stock emulsion containing 1.13 mmol triolein, 60 mg phosphatidylcholine, and 9 ml glycerol was prepared (20). A mixture of 1 volume of the stock emulsion, 19 vol of 3% (w/v) BSA in 0.2 M Tris/HCl buffer (pH 8.2), and 5 vol of heat inactivated (56°C, 10 min) serum from starved rats was incubated at 37°C for 15–30 min. For assay, 100 μl of this activated substrate mixture was added to 100 μl of the diluted extract of the powder, and the mixture was incubated for 30 min at 37°C. The reaction was terminated by adding 3 ml of a 1:1 (v/v) mixture of chloroform and heptane containing 2% (v/v) methanol. The free fatty acids produced were measured as described previously (21). Briefly, the mixture was shaken for 10 min and centrifuged at 2,000 × *g* for 5 min. The upper aqueous phase was removed by suction, and 1 ml of copper reagent prepared by the method of Zepf (22) was added to the lower organic phase. The mixture was shaken for 10 min and centrifuged at 2,000 × *g* for 10 min. An aliquot of the upper organic phase was mixed with the same volume of 0.1% (w/v) bathocuproin in chloroform containing 0.05% (w/v) 3-*tert*-butyl-4-hydroxyanisole and its absorbance was measured at 480 nm. One milliunit of lipolytic activity was defined as that releasing 1 nmol of fatty acid/min at 37°C.

Assay of glycerol-3-phosphate dehydrogenase activity

The cells were washed once with ice-cold PBS, harvested in ice-cold 25 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA, and sonicated briefly at 0°C. The homogenate was centrifuged at 8,000 × *g* for 20 min at 4°C. The supernatant was assayed for glycerol-3-phosphate dehydrogenase (GPDH) activity at 23°C by measuring the oxidation of NADH in the presence of dihydroxyacetone phosphate as described by Kozak and Jensen (23) and modified by Wise and Green (24).

DNA replication assay by [^{3}H]thymidine incorporation

Confluent 3T3-L1 fibroblasts were cultured in the presence of 20 μg/ml BPA for 2 days. The medium was then replaced with a culture medium containing either 5 μg/ml INS alone or a combination of 5 μg/ml INS and 20 μg/ml BPA. Three days later, the plates were replenished with a fresh medium containing the appropriate additive and incubated for 30 min. Then, 1 μCi of [^{3}H]thymidine (25.0 Ci/mmol) was added to each plate. Thirty minutes later, the cells were harvested in 1.8 ml of PBS containing 0.1% SDS and 1 mM EDTA. Measurement of acid-insoluble radioactivity was performed by trichloroacetic acid precipitation as described by Smulson et al. (25).

Western blot analysis of proliferating cell nuclear antigen

An aliquot of diluted extracts (19.6 μg DNA/ml) of acetone/ether powders was mixed with an equal volume of 0.125 M Tris/HCl buffer (pH 6.8) containing 4% (w/v) SDS, 10% (v/v) 2-mercaptoethanol, 20% (v/v) glycerol, and 0.002% (w/v) bromophenol blue, and heated for 5 min at 95°C. Proteins in the extracts were separated by SDS-PAGE in a Laemmli type system (26) with 15% (w/v) acrylamide resolving gel and 3% (w/v) acrylamide stacking gel. The separated proteins were then transferred electrophoretically to a PVDF membrane. Nonspecific binding was blocked by incubating the membranes with 5% (w/v) skim milk for 1 h. The blot was then incubated for 1 h with mouse monoclonal antibody to proliferating cell nuclear antigen (PCNA). Immunoreactivity was visualized with alkaline phosphatase-conjugated goat anti-mouse IgG and ECF substrate, and the enhanced chemifluorescence intensity was detected using a FluorImager, Fluorescence Imaging Analyzer (Amersham Pharmacia Biotech).

Chemical analysis

DNA was measured fluorometrically by the method of Hinegardner (27) using calf thymus DNA as standard. TG was measured using a kit for TG.

Statistical analysis

A Student's *t*-test was used to compare mean values. For all the statistical analyses, the criterion of significance was $P < 0.05$.

RESULTS

Dose-response relationship between BPA and LPL activity

The confluent cultures of 3T3-L1 fibroblasts were treated with 0–100 μg/ml BPA for 2 days and subse-

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quently treated with a combination of 5 μg/ml INS and BPA at the corresponding concentrations for 9 days. When BPA was absent throughout the 11 days, the cultures contained 176 mU/mg DNA of LPL activity. The addition of BPA to the cultures caused a dose-dependent increase in the LPL activity (**Fig. 1**). The presence of 2 μg/ml BPA caused a considerable increase in the LPL activity. BPA at 10 and 20 μg/ml increased the LPL activity by 170% and 560%, respectively. However, BPA at the concentrations of >40 μg/ml was toxic to these cells. Based on these results, the following experiments were performed using 20 μg/ml BPA.

Can BPA trigger 3T3-L1 fibroblasts to differentiate into adipocytes?

Whether BPA by itself can enhance the expression of phenotypic markers was determined. When no additive was present throughout the 11 days after confluence, the cultures contained 1.7 μg/μg DNA of TG and 149 mU/mg DNA of LPL activity. The presence of BPA alone during this period caused a 46% decrease in the TG content, but a 106% increase in the LPL activity (**Fig. 2**). The percentage of lipid-positive cells in the cultures was less than 2% (data not shown).

Next, the confluent cultures were treated with or without BPA for 2 days and subsequently treated with INS alone for 9 days. When BPA was absent during the first 2-day treatment period, the cultures contained 1.6 μg/μg DNA of TG, 202 mU/mg DNA of LPL activity, and 462 nmol/min/mg DNA of GPDH activity (**Table 1**). Numerous small lipid droplets were observed surrounding the nucleus, but the percentage of lipid-positive cells in the cultures was less than 10% (**Fig. 3A**). The presence of BPA during this period caused a 150% increase in the TG content, a 60% increase in the LPL activity, and a 500% increase in the GPDH activity (Table 1). The lipid droplets coalesced and became larger, and the intensity of staining of the individual cells increased (Fig. 3B). The percentage of lipid-positive cells in the cultures also increased to approximately 28%. Thus, the 2-day treatment of confluent cultures with BPA enhanced the expression of phenotypic markers.

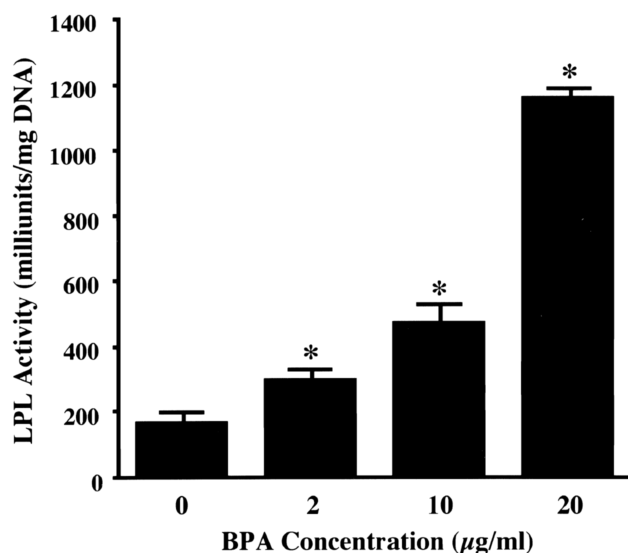

Fig. 1. Dose-response relationship between PBA and LPL activity. The confluent cultures were treated with bisphenol A (BPA) at the indicated concentrations for 2 days and subsequently treated with a combination of 5 μg/ml insulin (INS) and BPA at the corresponding concentrations for 9 days. The cells were harvested, sonicated briefly at 0°C, and used to make acetone/ether powders. LPL activity in aqueous extracts of the powders was measured. Values given are the mean ± SD for four plates. $^*P < 0.01$ (compared with the value obtained from the cultures treated in the absence of BPA throughout 11 days).

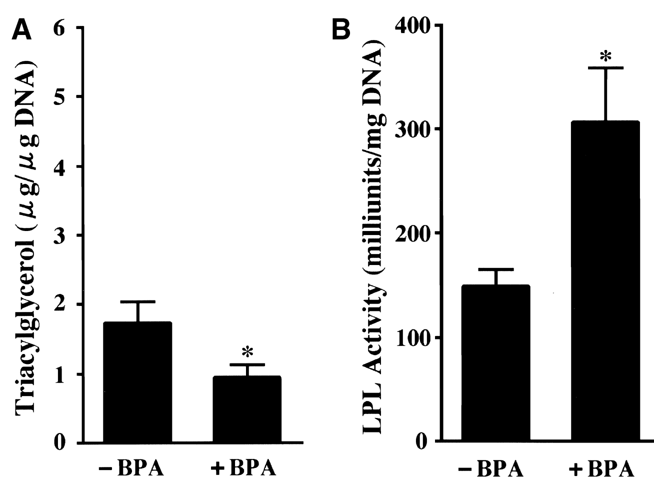

Fig. 2. Effect of BPA on triacylglycerol (TG) content and LPL activity. The confluent cultures of 3T3-L1 fibroblasts were treated for 11 days without or with 20 μg/ml BPA. The cells were harvested and sonicated briefly at 0°C. A: An aliquot of the homogenate was used to measure TG content. B: Another aliquot was used to make an acetone/ether powder and LPL activity in aqueous extracts of the powders was measured. The DNA content of the cultures treated without BPA was 39.1 ± 2.3 μg/plate and that of the cultures treated with BPA was 22.1 ± 1.6 μg/plate. Values given are the mean ± SD for four plates. $^*P < 0.01$ (compared with the value obtained from the cultures treated in the absence of BPA throughout 11 days).

Can BPA accelerate adipocyte conversion?

The confluent cultures were treated with BPA alone for 2 days and subsequently treated with INS in the absence or presence of BPA for 9 days. When no additive was present during the latter 9-day treatment period, the cultures contained 1.2 μg/μg DNA of TG and 502 mU/mg DNA of LPL activity. The presence of INS alone during this period caused a 200% increase in the TG content, but did not alter the LPL activity, while the simultaneous presence of BPA with INS caused a 1,300% increase in the TG content and a 190% increase in the LPL activity (**Table 2**). The GPDH activity of the cultures treated with a combination of INS and BPA also was 3.3-times higher than that of the cultures treated with INS alone (Table 1). The percentage of lipid-positive cells in the cultures was approximately 83% (Fig. 3C). These molecular and morphological properties of the cultures were very similar to those of the positive control cultures, which were treated with a combina-

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TABLE 1. Effect of BPA on triggering the differentiation of 3T3-L1 fibroblasts into adipocytes

Trigger	Treatment	DNA Content	TG Content	LPL Activity	GPDH Activity
		μg/plate	*μg/μg DNA*	*mU/DNA*	*nmol/min/mg DNA*
Exp. I					
No additive	INS	93.0 ± 2.6	1.63 ± 0.09	201.9 ± 38.8	n.d.[a]
BPA	INS	82.4 ± 7.8	4.09 ± 0.28*	324.1 ± 46.7**	n.d.
Exp. II					
No additive	INS	51.3 ± 1.8	n.d.	n.d.	462 ± 20
BPA	INS	53.1 ± 1.4	n.d.	n.d.	2769 ± 282*
BPA	INS+BPA	38.9 ± 0.5*	n.d.	n.d.	9027 ± 1834**

The confluent cultures of 3T3-L1 fibroblasts were treated with or without 20 μg/ml BPA for 2 days and subsequently treated with either 5 μg/ml INS or a combination of 5 μg/ml INS and 20 μg/ml BPA for 9 days.

Exp. I. The cells were harvested in ice-cold 50 mM NH_4Cl/NH_4OH buffer (pH 8.2) containing 2% (w/v) BSA and 20 μg/ml heparin and sonicated briefly at 0°C. Aliquots of the homogenate were used to measure DNA and TG contents and another aliquot was used to make an acetone/ether powder. LPL activity in aqueous extracts of the powders was measured. Values given are the mean ± SD for four plates.

Exp. II. The cells were harvested in ice-cold 25 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA, and sonicated briefly at 0°C. The homogenate was centrifuged at 8,000 *g* for 20 min at 4°C. The supernatant was assayed for GPDH activity at 23°C by measuring the oxidation of NADH in the presence of dihydroxyacetone phosphate. Values given are the mean ± SD for three plates.

[a] n.d., not determined.

* $P < 0.01$, ** $P < 0.05$ (compared with the value obtained from the cultures treated without BPA during the first 2-day treatment period).

tion of INS, DEX, and MIX for 2 days and subsequently treated with INS alone for 9 days (Table 2 and Fig. 3D).

The TG content and LPL activity in the cultures treated with a combination of INS, DEX, and MIX in the presence of BPA during the first 2-day treatment period were 50% and 47%, respectively, of those in the positive control cultures (Table 2).

The DNA content of the cultures in which no additive was present during the latter 9-day treatment period was 37 μg/plate. The presence of INS alone during this period caused an 80% increase in the DNA content, but the simultaneous presence of BPA with INS did not change it (Table 2).

Effect of chemicals relevant to BPA on adipocyte conversion

The confluent cultures were treated with a chemical relevant to BPA for 2 days and subsequently treated with a combination of INS and the corresponding chemical. The presence of either BPA or BPADA in combination with INS caused a 194% and 62%, respectively, increase in the TG content and a 115% and 30%, respectively, increase in the LPL activity, compared with those of the cultures treated with BPA for 2 days followed by the 9-day treatment with INS alone (**Table 3**). The presence of a combination of INS and BPABCF caused a 53% increase in the TG content but did not alter the LPL activity, but the presence of a combination of INS and BPADGE altered neither the TG content nor LPL activity.

BPADA and BPABCF caused a 25% and 31%, respectively, decrease in the DNA content similar to BPA (Table 3). BPADGE did not affect the DNA content.

Time courses of cell proliferation, TG accumulation, and development of LPL activity

The DNA content of the cultures was measured to monitor cell proliferation. The confluent cultures (day 0) contained 28 μg/plate of DNA. The DNA content of the cultures did not change during the 2-day treatment of the confluent cultures with BPA alone. It then increased slightly during the first 5-day treatment with a combination of INS and BPA and increased to 45 μg/plate during the next 4-day treatment period (**Fig. 4A**). In the positive control cultures, the DNA content increased to 39 μg/plate during the 2-day treatment of the confluent cultures

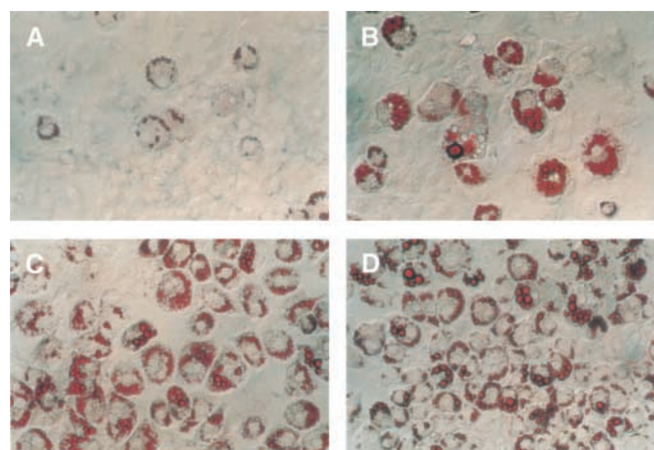

Fig. 3. Oil Red O staining of 3T3-L1 adipocytes. A: The confluent cultures were treated in the absence of BPA for 2 days and subsequently treated with 5 μg/ml INS alone for 9 days. B: The confluent cultures were treated with 20 μg/ml BPA for 2 days and subsequently treated with 5 μg/ml INS alone for 9 days. C: The confluent cultures were treated with 20 μg/ml BPA for 2 days and subsequently treated with a combination of 5 μg/ml INS and 20 μg/ml BPA for 9 days. D: The confluent cultures were treated with a combination of 10 μg/ml INS, 1 μM DEX, and 0.5 mM MIX for 2 days, and subsequently treated with 5 μg/ml INS alone for 9 days. At the end of the experiments, the cells were fixed with 10% formalin and then stained with Oil Red O. The representative of three plates is shown. Magnification ×100.

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TABLE 2. Effect of BPA alone, INS alone, and a combination of INS and BPA on adipocyte conversion

Trigger	Treatment	DNA Content	TG Content	LPL Activity
		μg/plate	*μg/μg DNA*	*mU/DNA*
BPA	No additive	37.0 ± 3.0	1.22 ± 0.10	502.3 ± 85.4
BPA	INS	66.4 ± 2.6*	3.66 ± 0.16*	480.6 ± 14.2
BPA	INS+BPA	37.8 ± 2.1	17.12 ± 2.11*	1442.3 ± 98.6*
INS+DEX+MIX	INS	56.2 ± 2.0*	13.50 ± 0.74*	1522.3 ± 201.9*
INS+DEX+MIX+BPA	INS	53.9 ± 1.8*	6.73 ± 0.93*	720.9 ± 198.9

The confluent cultures of 3T3-L1 fibroblasts were treated for 2 days with 20 μg/ml BPA and subsequently treated for 9 days with the following additive; *i*) no additive, *ii*) 5 μg/ml INS alone, *iii*) 5 μg/ml INS plus 20 μg/ml BPA. In another series of experiments, the confluent cultures were treated for 2 days with a combination of 10 μg/ml INS, 1 μM DEX, and 0.5 mM MIX in the absence or presence of 20 μg/ml BPA and subsequently treated for 9 days with 5 μg/ml INS alone. The cells were harvested and sonicated briefly at 0°C. Aliquots of the homogenate were used to measure DNA and TG contents and another aliquot was used to make an acetone/ether powder. LPL activity in aqueous extracts of the powders was measured. Values given are the mean ± SD for four plates.

* $P < 0.01$ (compared with the value obtained from the cultures in which no additive was present during the latter 9-day treatment period).

with a combination of INS, DEX, and MIX, and increased linearly to 62 μg/plate during the subsequent 5-day treatment with INS alone, and then became a plateau (Fig. 4A).

Next, the expression of PCNA in cells, which appears in the nuclei of proliferating cells (28–33), was estimated by a Western blot analysis (**Fig. 5**). In the cells of the confluent cultures (day 0), a mouse monoclonal antibody to PCNA identified a band corresponding to PCNA (Mr = 36,000). In the positive control cultures, the expression of PCNA on day 2 was slightly higher than that on day 0, but then it became lower. The band on day 11 was very faint. In the cultures treated with BPA for 2 days followed by the 9-day treatment with a combination of INS and BPA, the band was detectable but was fainter than that on the corresponding days of the positive control cultures.

The TG content of the cultures was 0.8 μg/μg DNA on day 0 and did not significantly change during the subsequent 2-day treatment with BPA alone. It increased slightly during the first 3-day treatment with a combination of INS and BPA and increased more than 16-fold to 16 μg/μg DNA during the next 6-day treatment period (Fig. 4B). The TG content of the positive control cultures also increased in a similar manner.

The LPL activity of the cultures was 11 mU/mg DNA on day 0 and increased slightly to 19 mU/mg DNA during the subsequent 2-day treatment with BPA alone. It increased more than 3-fold to 88 mU/mg DNA during the first 3-day treatment with a combination of INS and BPA and increased more than 40-fold to 845 mU/mg DNA during the next 6-day treatment period (Fig. 4C). The LPL activity of the positive control cultures also developed in a similar manner.

TABLE 3. Effect of chemicals relevant to BPA on adipocyte conversion

Trigger	Treatment	DNA Content	TG Content	LPL Activity
		μg/plate	*μg/μg DNA*	*mU/DNA*
BPA	INS	59.0 ± 2.2	3.07 ± 0.06	472.8 ± 7.5
BPA	INS+BPA	41.1 ± 3.8*	9.04 ± 0.51*	1015.9 ± 35.8*
BPADA	INS+BPADA	44.5 ± 1.9*	4.99 ± 0.12*	616.7 ± 19.1*
BPABCF	INS+BPABCF	41.0 ± 0.9*	4.69 ± 0.17*	563.2 ± 65.3
BPADGE	INS+BPADGE	55.2 ± 2.0	3.54 ± 0.30	497.7 ± 55.4

The confluent cultures of 3T3-L1 fibroblasts were treated with 20 μg/ml chemicals for 2 days and subsequently treated with either 5 μg/ml INS alone or a combination of 5 μg/ml INS and 20 μg/ml of the corresponding chemicals for 9 days. Then cells were harvested and sonicated briefly at 0°C. The DNA and TG contents in aliquots of the homogenates were measured. Another aliquot was used to make an acetone/ether powder. LPL activity in aqueous extract of the powder was measured. Values given are the mean ± SD for three plates.

BPADA, bisphenol A diacetate; BPABCF, bisphenol A bis(chloroformate); BPADGE, bisphenol A diglycidyl ether.

* $P < 0.01$ (compared with the value obtained from the cultures treated with BPA for 2 days followed by the 9-day treatment with INS alone).

Effect of BPA on DNA replication

The confluent cultures, which had been treated with BPA for 2 days and subsequently treated with either INS alone or a combination of INS and BPA for 3 days, were incubated for 30 min with [^{3}H]thymidine and the amount of radioactivity incorporated into DNA was measured. In the cultures treated with INS alone during the latter 3-day treatment period, the amount of ^{3}H in DNA was 8,927 cmp/plate and the DNA content of the cultures was 62.6 μg/plate (**Table 4**). The simultaneous presence of BPA with INS during this period caused a 46% decrease in the amount of ^{3}H in DNA and a 12% decrease, but not significantly, in the DNA content of the cultures. The ratios of the amount of ^{3}H in DNA to the DNA content of the cultures were 142 cpm/μg DNA in the cultures treated with INS alone and 86 cpm/μg DNA in the cultures treated with a combination of INS and BPA. This result indicates that BPA inhibited DNA replication.

DISCUSSION

In the present study, we showed that the environmental endocrine disrupting chemicals, especially BPA, in combination with INS can accelerate the conversion of 3T3-L1 fibroblasts to adipocytes. The chronological events occurring during the conversion of fibroblasts to adipocytes have been investigated in cultures (34). The time course of the changes

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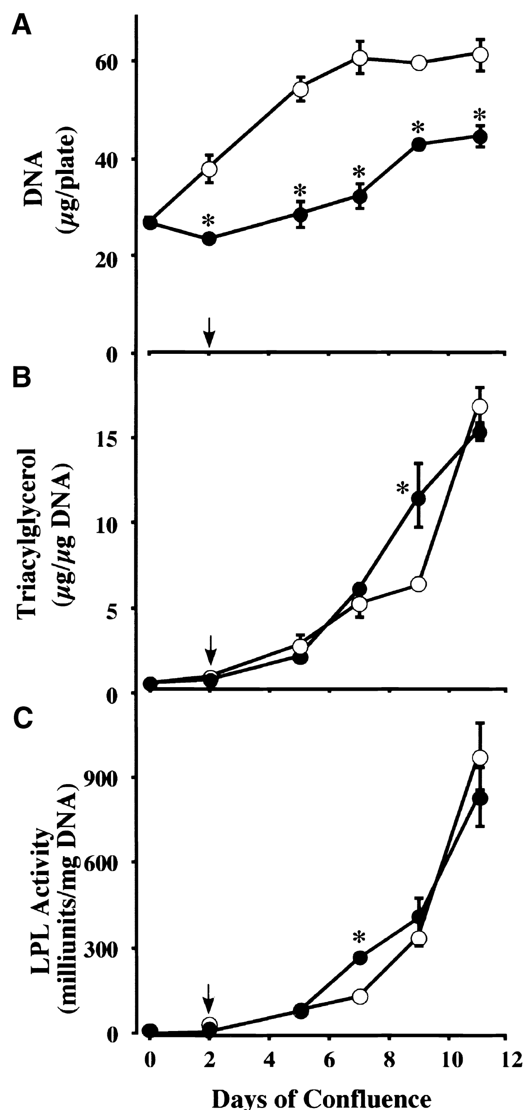

Fig. 4. Changes in DNA (A) and TG (B) contents and LPL activity (C) during adipocyte conversion. The confluent cultures (day 0) were treated with 20 µg/ml BPA for 2 days (closed circle). On day 2 (arrow), the medium was replaced with the medium containing a combination of 5 µg/ml INS and 20 µg/ml BPA and changed every 2 days. The confluent cultures were treated with a combination of 10 µg/ml INS, 1 µM DEX, and 0.5 mM MIX (open circle). On day 2, the medium was replaced with the medium containing 5µg/ml INS alone and changed every 2 days. At the indicated intervals, cells were harvested and sonicated briefly at 0°C. Aliquots of the homogenates were used for measurements of DNA and TG. Another aliquot was used to make acetone/ether powders and then LPL activity in aqueous extracts of the powders was measured. Values given are the mean ± SD for three plates. $*P < 0.01$ (compared with the value in the positive control cultures).

in phenotypic markers during adipocyte conversion is as follows: *i*) LPL appears early at confluence before TG accumulation in cells occurs; *ii*) GPDH appears later and TG accumulation occurs. Once 3T3-L1 fibroblasts reach confluence, they become epithelioid in shape and synthesize LPL within 1 day after confluence (13, 35). In this study, we used two criteria for adipocyte conversion; *i*) expression of LPL and GPDH activities and *ii*) TG accumulation in cells.

Although fibroblasts are converted spontaneously to adipocytes after confluence, this process takes 2–4 weeks (10). A variety of compounds that can retract the conversion process by rapidly and irreversibly triggering fibroblasts to differentiate into adipocytes have been reported (12–18). In the first set of experiments, we determined whether BPA was able to trigger the differentiation of fibroblasts into adipocytes. When BPA was absent during the 2-day treatment of confluent cultures, not only were the TG content and LPL and GPDH activities of the cultures low (Table 1), but also the lipid droplets in individual cells remained small (Fig. 3A), regardless of the presence of INS alone during the subsequent 9-day treatment period. However, the presence of BPA during the first 2-day treatment period caused an increase in the TG content from 1.6 to 4.1 µg/µg DNA, an increase in the LPL activity from 202 to 324 mU/mg DNA, and an increase in the GPDH activity from 462 to 2,769 nmol/min/mg DNA (Table 1). In addition, the lipid droplets in the individual cells of these cultures coalesced and became larger with time, and the percentage of lipid-positive cells in the cultures also increased from <10% to 28% (Fig. 3B). Thus, the 2-day treatment of confluent cultures with BPA stimulated the expression of phenotypic markers. These results are similar to the finding of Russell and Ho (15) that short-term (1 to 2 days) treatment of confluent 3T3-L1 fibroblasts with either MIX or PGF2α stimulated lipid accumulation in cells cultured subsequently with INS alone. They concluded that these reagents triggered the differentiation process by rapidly programming 3T3-L1 fibroblasts to differentiate into adipocytes. Although the most effective trigger is a combination of INS, DEX, and MIX, the continuous presence of this combination is not required for lipid accumulation (14, 17, 18). Based on these findings, we concluded that BPA had the ability to trigger the differentiation of 3T3-L1 fibroblasts into adipocytes.

In the second set of experiments, we determined whether BPA was able to stimulate the adipocyte conversion process. Although INS alone stimulated the expression of phenotypic markers in the cultures treated with BPA during the first 2-day treatment period, the levels of the TG content and LPL activity of such cultures were 27% and 32%, respectively, of those of the positive control cultures (Table 2). The appearance of lipid-positive cells in the cultures also was about one third of that in the positive control cultures (Fig. 3B); however, the simultaneous presence of BPA with INS, instead of INS alone, caused an increase in the TG content from 3.7 to 17.1 µg/µg DNA and an increase in the LPL activity from 481 to 1,442 mU/mg DNA (Table 2). The percentage of lipid-positive cells in the cultures also increased from 28% to 83% (Fig. 3C). These molecular and morphological properties of the cultures were very similar to those of the positive control cultures (Table 2 and Fig. 3D). Similarly, the GPDH activity was higher in the cultures treated with a combination of INS and BPA than in those treated with INS alone (Table 1). These results indicate that BPA in combination with INS can accelerate the adipocyte conversion. Other chemicals relevant to BPA, except for BPADGE, also had a simi-

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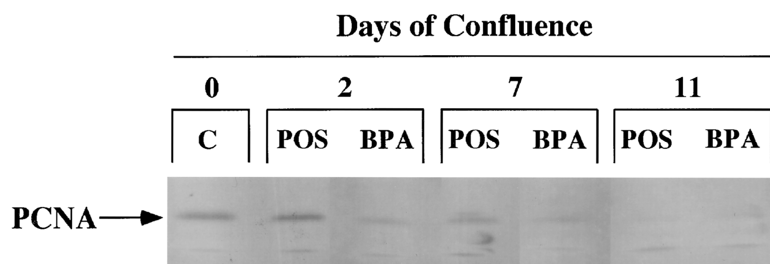

Fig. 5. Western blot of proliferating cell nuclear antigen (PCNA). The Western blot analysis of PCNA was performed in the extracts of acetone/ether powders of cells prepared in Fig. 4. Samples containing 30 ng DNA were loaded to each lane on SDS-PAGE. The representative of three experiments is shown. C, the confluent cultures; POS, the positive control cultures; BPA, the cultures treated with BPA for 2 days after confluence followed by the 9-day treatment with a combination of INS and BPA.

lar effect on adipocyte conversion (Table 3). The order of their potency to stimulate the expression of phenotypic markers was as follows: BPA >> BPADA > BPABCF.

3T3-L1 fibroblasts have been reported to be capable of converting to adipocytes after they stop proliferating (10–12, 16). Therefore, in the final set of experiments, we examined the effect of BPA on cellular proliferation. 3T3-L1 cells replicated at a lower proliferative rate when they were cultured in the presence of BPA (Fig. 4A). Table 2 showed that BPA inhibited the increasing effect of INS on the DNA content of the cultures. Other chemicals, except for BPADGE, also had a similar effect on the DNA content (Table 3). These findings suggest that the environmental endocrine disrupting chemicals, which had the ability to stimulate the expression of phenotypic markers, had an anti-proliferative activity on 3T3-L1 cells. The finding that the amount of [^{3}H]thymidine incorporated into DNA was lower in the cultures treated with a combination of INS and BPA than in those treated with INS alone confirms it (Table 4).

A Western blot analysis of PCNA showed that BPA decreased PCNA production in cells (Fig. 5). Expression of PCNA in cells is closely linked to the cell cycle (28, 29). The level of PCNA in the nucleus begins to increase during the late G1 phase immediately before the onset of DNA synthesis, becomes maximal during the S phase, and decreases again during the G2 and M phases. Therefore, PCNA is often used as a marker of proliferating cells (28–33). Taken together, our results indicate that BPA inhibits DNA synthesis by arresting 3T3-L1 cells in the G1 phase.

TABLE 4. Effect of BPA on the incorporation of [^{3}H]thymidine into DNA

Trigger	Treatment	DNA Content	[^{3}H]thymidine Incorporated into DNA
		μg/plate	*cpm/plate*
BPA	INS	62.6 ± 1.1	8927 ± 617
BPA	INS+BPA	55.4 ± 3.1	4853 ± 139*

The confluent cultures of 3T3-L1 fibroblasts were treated with 20 μg/ml BPA for 2 days and subsequently treated with either 5 μg/ml INS alone or a combination of 5 μg/ml INS and 20 μg/ml BPA for 3 days. The cells were pulse-labeled for 30 min with [^{3}H]thymidine, and the acid-insoluble radioactivity was then measured by trichloroacetic acid precipitation and liquid scintillation counting. In this experiment, we used six plates for each treatment and divided them into two groups. Three plates were used for the [^{3}H]thymidine incorporation study and the others were used for the DNA measurement. Values given are the mean ± SD for three plates.

* $P < 0.01$ (compared with the value obtained from the cultures treated with INS alone during the latter 3-day treatment period).

The reentry of growth-arrested 3T3-L1 fibroblasts into cell cycle, termed mitotic clonal expansion, has been reported to be necessary for optimal adipocyte differentiation (36, 37). A combination of INS, DEX, and MIX, the most effective trigger of the differentiation, induces reentry of these cells into the cell cycle. The simultaneous presence of BPA with this combination during the first 2-day treatment period of the confluent cultures caused a marked decrease in the expression of phenotypic markers compared with the positive control cultures (Table 2). This result suggests that BPA might inhibit the combination-induced mitotic clonal expansion. However, a possibility that BPA had the ability to induce reentry of growth-arrested 3T3-L1 fibroblasts into the cell cycle cannot be excluded, because BPA has been reported to increase the expression of c-Fos gene, a growth-associated gene, in the uterus and vagina of ovariectomized rats (38).

The TG content of the cultures treated with BPA alone throughout 11 days after confluence was very low (Fig. 2), indicating that BPA by itself did not have the ability to stimulate TG accumulation in cells. However, BPA potentiated the ability of INS to accumulate TG in the cells capable of expressing the differentiated phenotype (Table 2). The mechanism by which BPA stimulated the INS-induced TG accumulation is unclear. INS increases the incorporation of glucose and fatty acids into TG in 3T3-L1 cells (12, 13). Mackall et al. (39) and Grimaldi et al. (40) reported that the activities of several enzymes involved in fatty acid synthesis increased in 3T3-L1 cells treated with INS after confluence. BPA may potentiate these INS-mediated metabolic activities to stimulate TG accumulation in cells. It is unlikely that the BPA-stimulated TG accumulation was as a result of the activation of peroxisome proliferator-activated receptor *r* (PPAR*r*), which is abundantly expressed in adipocytes and function as a key regulator of adipocyte conversion (9, 41–44), by BPA, because BPA has been reported to be unable to bind to this receptor (9). However, a possibility that BPA modulates PPAR*r* gene expression, directly or indirectly, cannot be excluded. Funabashi et al. (45) reported that administration of BPA to ovariectomized rats caused an increase in progesterone receptor mRNA level in mediobasal hypothalamus and anterior pituitary.

Obesity is one of the greatest concerns in public health. Obesity is the result of an increase in body fat mass produced by either an enlargement of fat cells (fat cell hypertrophy) or an increased number of these cells (fat cell hyperplasia) or both. Since BPA was able to enter fibroblasts in the differentiation process and enhance the adipocyte con-

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version in combination with INS, this suggests that in vivo prolonged exposure to BPA might increase body fat mass and involve the development of obesity. BPA is present ubiquitously in the environment. For example, BPA is used commercially in the products of polycarbonate plastics and leaches from them when subjected to high temperatures (4). Microgram amounts of BPA have also been found in the liquid of preserved vegetables in cans (3) and in the saliva of patients treated with dental sealants (5). Thus, humans have been exposed chronically to BPA. Therefore, it will be important to examine the relationship between chronic exposure to BPA and development of obesity.

This work was supported in part by Grant-in-Aid for Scientific Research (C) from Japan Society for the Promotion of Science (H.M.).

Manuscript received 21 August 2001 and in revised form 15 January 2002.

REFERENCES

1. Fry, D. M. 1995. Reproductive effects in birds exposed to pesticides and industrial chemicals. *Environ. Health Perspect.* **103:** 165–171.
2. Guillette, Jr., L. J., D. A. Crain, A. A. Rooney, and D. B. Pickford. 1995. Organization vs. Activation: the role of endocrine-disrupting contaminants (EDCs) during embryonic development in wildlife. *Environ. Health Perspect.* **103:** 157–164.
3. Brotons, J. A., M. F. Olea-Serrano, M. Villalobos, V. Pedraza, and N. Olea. 1995. Xenoestrogens released from lacquer coatings in food cans. *Environ. Health Perspect.* **103:** 608–612.
4. Krishnan, A. V., P. Stathis, S. F. Permuth, L. Tokes, and D. Feldman. 1993. Bisphenol-A: an estrogenic substance is released from polycarbonate flasks during autoclaving. *Endocrinology.* **132:** 2279–2286.
5. Olea, N., R. Pulgar, P. Perez, M. F. Olea-Serrano, A. Rivas, A. Novillo-Fertrell, V. Pedraza, A. M. Soto, and C. Sonnenschein. 1996. Estrogenicity of resin-based composites and sealants used in dentistry. *Environ. Health Perspect.* **104:** 298–305.
6. Korach, K. S. 1993. Editorial: surprising places of estrogenic activity. *Endocrinology.* **132:** 2277–2278.
7. Wade, G. N., and J. M. Gray. 1979. Gonadal effects on food intake and adiposity: a metabolic hypothesis. *Physiol. Behave.* **22:** 583–593.
8. Benoit, V., A. Valette, L. Mercier, J. M. Meignen, and J. Boyer. 1982. Potentiation of epinephrine-induced lipolysis in fat cells from estrogen-treated rats. *Biochem. Biophys. Res. Commun.* **109:** 1186–1191.
9. Wright, H. M., C. B. Clish, T. Mikami, S. Hauser, K. Yanagi, R. Hiramatsu, C. N. Serhan, and B. M. Spiegelman. 2000. A synthetic antagonist for the peroxisome proliferator-activated receptor *r* inhibits adipocyte differentiation. *J. Biol. Chem.* **275:** 1873–1877.
10. Green, H., and M. Meuth. 1974. An established pre-adipose cell line and its differentiation in culture. *Cell.* **3:** 127–133.
11. Green, H., and O. Keihinde. 1975. An established pre-adipose cell line and its differentiation in culture. II. Factors affecting the adipose conversion. *Cell.* **5:** 19–27.
12. Green, H., and O. Keihinde. 1976. Spontaneous heritable changes leading to increased adipose conversion in 3T3 cells. *Cell.* **7:** 105–113.
13. Spooner, P. M., S. S. Chernick, M. M. Garrison, and R. O. Scow. 1979. Development of lipoprotein lipase activity and accumulation of triacylglycerol in differentiating 3T3–L1 adipocytes. Effects of prostaglandin F2α, 1-methyl-3- isobutylxanthine, prolactin, and insulin. *J. Biol. Chem.* **254:** 1305–1311.
14. Rubin, C. S., A. Hirsch, C. Fung, and O. M. Rosen. 1978. Development of hormone receptors and hormonal responsiveness in vitro. Insulin receptors and insulin sensitivity in the preadipocyte and adipocyte forms of 3T3–L1 cells. *J. Biol. Chem.* **253:** 7570–7579.
15. Russell, T. R., and R-H. Ho. 1976. Conversion of 3T3–L1 fibroblasts into adipose cells: triggering of differentiation by prostaglandin F2α and 1-methyl-3-isobutylxanthine. *Proc. Natl. Acad. Sci. USA.* **73:** 4516–4520.
16. Toscani, A., D. R. Soprano, and K. J. Soprano. 1990. Sodium butyrate in combination with insulin or dexamethasone can terminally differentiate actively proliferating Swiss 3T3–L1 cells into adipocytes. *J. Biol. Chem.* **265:** 5722–5730.
17. Reed, B. C., and M. D. Lane. 1980. Insulin receptor synthesis and turnover in differentiating 3T3–L1 preadipocytes. *Proc. Natl. Acad. Sci. USA.* **77:** 285–289.
18. Bernlohr, D. A., C. W. Angus, M. D. Lane, M. A. Bolanowski, and T. J. Kelly, Jr. 1984. Expression of specific mRNAs during adipose differentiation: identification of an mRNA encoding a homologue of myelin P2 protein. *Proc. Natl. Acad. Sci. USA.* **81:** 5468–5472.
19. Kuri-Harcuch, W., and H. Green. 1978. Adipose conversion of 3T3 cells depends on a serum factor. *Proc. Natl. Acad. Sci. USA.* **75:** 6107–6109.
20. Masuno, H., E. J. Blanchette-Mackie, S. S. Chernick, and R. O. Scow. 1990. Synthesis of inactive nonsecretable high mannose-type lipoprotein lipase by cultured brown adipocytes of combined lipase-deficient *cld/cld* mice. *J. Biol. Chem.* **265:** 1628–1638.
21. Masuno, H., T. Ohara, A. Ide, and H. Okuda. 1984. Correlation of lipolytic and lipogenic actions of synthetic peptides with structure. *J. Biochem.* **95:** 1083–1090.
22. Zepf, J., E. Schoenle, M. Waldvogel, I. Sand, and E. R. Froesch. 1981. Effect of trypsin treatment of rat adipocytes on biological effects and binding of insulin and insulin-like growth factors: further evidence for the action of insulin-like growth factors through the insulin receptor. *Eur. J. Biochem.* **113:** 605–609.
23. Kazak, L. P., and J. T. Jensen. 1974. Genetic and developmental control of multiple forms of L-glycerol-3-phosphate dehydrogenase. *J. Biol. Chem.* **249:** 7775–7781.
24. Wise, L. S., and H. Green. 1979. Participation of one isozyme of cytosolic glycerophosphate dehydrogenase in the adipose conversion of 3T3 cells. *J. Biol. Chem.* **254:** 273–275.
25. Smulson, M. E., V. H. Kang, J. M. Ntambi, D. S. Rosenthal, R. Ding, and C. M. G. Simbulan. 1995. Requirement for the expression of poly(ADP-ribose) polymerase during the early stages of differentiation of 3T3–L1 preadipocytes, as studied by antisense RNA induction. *J. Biol. Chem.* **270:** 119–127.
26. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **227:** 680–685.
27. Hinegardner, R. T. 1971. An improved fluorometric assay for DNA. *Anal. Biochem.* **39:** 197–201.
28. Takasaki, Y., J. S. Deng, and E. M. Tan. 1981. A nuclear antigen associated with cell proliferation and blast transformation. *J. Exp. Med.* **154:** 1899–1909.
29. Celis, J. E., and A. Celis. 1985. Cell cycle-dependent variations in the distribution of the nuclear protein cyclin proliferating cell nuclear antigen in cultured cells: subdivision of S phase. *Proc. Natl. Acad. Sci. USA.* **82:** 3262–3266.
30. Robbins, B. A., D. de la Vega, K. Ogata, E. M. Tan, and R. M. Nakamura. 1987. Immunohistochemical detection of proliferating cell nuclear antigen in solid human malignancies. *Arch. Pathol. Lab. Med.* **111:** 841–845.
31. Matsuno, Y., S. Hirohashi, S. Furuya, M. Sakamoto, K. Mukai, and Y. Shimosato. 1990. Heterogeneity of proliferative activity in nodule-in-nodule lesions of small hepatocellular carcinoma. *Jpn. J. Cancer Res.* **81:** 1137–1140.
32. Sakayama, K., H. Masuno, T. Miyazaki, H. Okumura, T. Shibata, and H. Okuda. 1994. Existence of lipoprotein lipase in human sarcomas and carcinomas. *Jpn. J. Cancer Res.* **85:** 515–521.
33. Sakayama, K., H. Masuno, H. Okumura, T. Shibata, and H. Okuda. 1996. Recombinant human tumor necrosis factor-α suppresses synthesis, activity and secretion of lipoprotein lipase in cultures of a human osteosarcoma cell line. *Biochem. J.* **316:** 813–817.
34. Ailhaud, G., E. Amri, D. Czerucka, C. Forest, D. Gaillard, P. Grimaldi, R. Negrel, and C. Vannier. 1985. Lipoprotein lipase and adipocyte differentiation. *Reprod. Nutr. Dev.* **25:** 153–158.
35. Eckel, R. H., W. Y. Fujimoto, and J. D. Brunzell. 1977. Development of lipoprotein lipase in cultured 3T3–L1 cells. *Biochem. Biophys. Res. Commun.* **78:** 288–293.
36. Cornelius, P., O. A. MacDougald, and M. D. Lane. 1994. Regulation of adipocyte development. *Annu. Rev. Nutr.* **14:** 99–129.
37. Patel, Y. M., and M. D. Lane. 2000. Mitotic clonal expansion during preadipocyte differentiation: calpain-mediated turnover of p27. *J. Biol. Chem.* **275:** 17653–17660.
38. Steinmetz, R., N. A. Mitchner, A. Grant, D. L. Allen, R. M. Bigsby, and N. Ben-Jonathan. 1998. The xenoestrogen bisphenol A induces growth, differentiation, and c-fos gene expression in the female reproductive tract. *Endocrinology.* **139:** 2741–2747.

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39. Mackall, C. J., A. K. Student, S. E. Polakis, and M. D. Lane. 1974. Induction of lipogenesis during differentiation in a “preadipocyte” cell line. *J. Biol. Chem.* **251:** 6462–6464.
40. Grimaldi, P., R. Negrel, and G. Ailhaud. 1978. Induction of the triglyceride pathway enzymes and of lipolytic enzymes during differentiation in a ‘preadipocyte’ cell line. *Eur. J. Biochem.* **84:** 369–376.
41. Tontonoz, P., E. Hu, and B. M. Spiegelman. 1994. Stimulation of adipogenesis in fibroblasts by PPAR*r*2, a lipid-activated transcription factor. *Cell.* **79:** 1147–1156.
42. Lehmann, J. M., L. B. Moore, T. A. Smith-Oliver, W. O. Wilkinson, T. M. Willson, and S. A. Kliewer. 1995. An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor *r*(PPAR*r*). *J. Biol. Chem.* **270:** 12953–12956.
43. Forman, B. M., P. Tontonoz, J. Chen, R. P. Brun, B. M. Spiegelman, and R. M. Evans. 1995. 15-Deoxy-Δ12,14-prostaglandin J2 is a ligand for the adipocyte determination factor PPAR*r*. *Cell.* **83:** 803–812.
44. Kliewer, S. A., J. M. Lenhard, T. M. Willson, I. Patel, D. C. Morris, and J. M. Lehmann. 1995. A prostaglandin J2 metabolite binds peroxisome proliferator-activated receptor *r* and promotes adipocyte differentiation. *Cell.* **83:** 813–819.
45. Funabashi, T., M. Kawaguchi, and F. Kimura. 2001. The endocrine disrupters butyl phthalate and bisphenol A increase the expression of progesterone receptor messenger ribonucleic acid in the preoptic area of adult ovariectomized rats. *Neuroendocrinology.* **74:** 77–81.

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