Attenuated Prostaglandin Formation in Peroxisomal-deficient Human Skin Fibroblasts

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Abstract

Peroxisomal-deficient skin fibroblasts from patients with Zellweger's syndrome or infantile Refsum's disease produced fewer prostaglandins than normal skin fibroblasts. Radioimmunoassay indicated a 45–55% decrease in prostaglandin E_2 (PGE_2) production when Zellweger's fibroblasts were incubated with arachidonic acid. This deficiency was not overcome by pretreatment of the Zellweger's fibroblasts with media containing arachidonic acid, and it was not due to channeling of arachidonic acid into other eicosanoid products. Modifications in the peroxide tone of the Zellweger's fibroblasts by addition of H_2O_2 or catalase failed to increase PGE_2 production. Using Northern analysis, we were unable to detect an mRNA transcript for PGH synthase in unstimulated Zellweger fibroblasts but identified a 4.2-kb mRNA transcript after treatment with phorbol myristate acetate (PMA). Treatment for 6 h with 10 nM PMA raised PGE_2 production in normal and Zellweger fibroblasts to equivalent levels. These increases were prevented by addition of H-7, staurosporine, cycloheximide, or actinomycin D. Our findings suggest that the reduced PGE_2 production in peroxisomal deficient fibroblasts is due to a decrease in PGH synthase mRNA. The reduction in PGH synthase can be overcome by treatment of the cells with agents which enhance gene expression. (*J. Clin. Invest.* 1993. 91:169–178.) Key words: phorbol ester • prostaglandins • protein kinase • Zellweger

Introduction

Peroxisomes are single membrane organelles 0.2–1.0 μm in diameter that are ubiquitous in eukaryotic cells (1). These organelles carry out a number of important biological functions. They contain catalase and thereby compartmentalize the detoxification of intracellular H_2O_2 (2), β-oxidize long chain and very long chain fatty acids (3), synthesize the alkyl ether bond of plasmalogens (4), produce bile acids (5), catabolize phytanic acid (6) and pipecolic acid (7), and are responsible for the chain-shortening of hydroxyeicosatetraenoic acids (8). The prototypical peroxisomal deficiency in humans has been designated the Zellweger or cerebrohepatorenal syndrome (1). This autosomal recessive disorder is characterized biochemically by the accumulation of phytanic acid and very long chain fatty acids, diminished plasmalogen synthesis, and decreased bile acid synthesis (9). Clinically, the syndrome is associated with failure to thrive, severe hypotonia, epileptic seizures, psychomotor retardation, hepatic and renal cortical cysts, and premature death in the first 6–12 mo of life (1). Liver and kidney tissue from infants with this syndrome have no demonstrable peroxisomes (10). Other human peroxisomal deficiencies have been described, including infantile Refsum's disease. This disease, although not identical to Zellweger's syndrome, shares many of the same biochemical and clinical features (1).

Prostaglandins (PGs) are important mediators of a number of critical functions in mammalian cells and tissues (11). The control of PG formation depends on the availability of arachidonic acid and the activity of PGH synthase, a membrane-bound heme protein that has two activities, a cyclooxygenase component and a peroxidase component (12). PGH synthase converts the arachidonic acid into an endoperoxide intermediate, PGH_2. Very little free arachidonic acid is found in cells; it is contained primarily in cellular phosphoglycerides and is released through the action of calcium-dependent phospholipases (13).

None of the biochemical defects in peroxisomal-deficient cells described to date suggests that PG formation is abnormal. We have observed, however, that agonist stimulated PG formation in peroxisomal-deficient skin fibroblasts is markedly reduced. The reduction in PGE_2 formation is not due to lack of substrate; it appears to be due to a reduced quantity of PGH synthase protein and mRNA transcription in the peroxisomal-deficient cells. Treatment of peroxisomal deficient cells with PMA restores PGH synthase enzyme activity, apparently through a pathway involving gene transcription and translation mediated by protein kinase C.

Methods

Cell culture. Zellweger and normal human skin fibroblasts were grown and maintained as previously described (8). The mutant skin fibroblasts, obtained from three different individuals, were supplied by the Pediatrics Cytogenetics Laboratory at the University of Iowa College of Medicine. Infantile Refsum's disease human skin fibroblasts were obtained from the Human Genetic Mutant Cell Repository (Camden, NJ). The cells were grown to confluency in Eagle's MEM containing 20% FBS (Hy-Clone Laboratories, Logan, UT), supplemented with nonessential amino acids, MEM vitamin solution (Gibco, Grand Island, NY), glutamine, and penicillin/streptomycin 100 μg/ml (Gibco). Cells were harvested with trypsin diluted 1:10 and passaged into tissue culture plates with a surface area of 5 or 10 cm^2/well (Tissue Culture Cluster 6 and 12; Costar, Cambridge, MA), or flasks with a surface area of 75 cm^2, (Corning Glass Works, Corning, NY). Experiments were performed when cell cultures were 80–100% confluent. Because of variability in growth rate and availability of cells, cultures were often studied at different times and passage number. Zellweger

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fibroblasts were studied between passages 3 and 7, infantile Refusum's disease fibroblasts between passages 9 and 11, and normal human skin fibroblasts between passages 18 and 24. Cultures were maintained in a temperature- and humidity-controlled incubator (CO_2 Incubator 3028; Forma Scientific, Inc., Marietta, OH) at 37°C with 95% air-5% CO_2 as the gas phase. The DNA content of the cells was measured fluorometrically (14).

Incubation and lipid analysis. After removal of the maintenance medium, the cells were washed twice with Dulbecco's PBS (DPBS)[1] containing 0.1 μM BSA and incubated for 20 min at 37°C in 1 ml of modified Eagle's MEM containing 7.5 μM [1-^{14}C]arachidonic acid and 0.1 μM BSA. After incubation, the medium was removed and placed in a siliconized glass tube, and the pH was adjusted to 3.5 by addition of 10 μl of 2.4 N HCl. Lipids were extracted three times with 2 vols of HPLC-grade ethyl acetate. The ethyl acetate extracts were pooled, evaporated to dryness under a stream of nitrogen, and resuspended in 500 μl of acetonitrile. The radioactive lipids were analyzed by reverse-phase HPLC (8).

Fatty acid methyl esters were prepared from the cell lipids extracted into chloroform/methanol solution (2:1, vol/vol) by incubation with boron trifluoride in methanol and acetonitrile for 30 min at 90°C. After extraction of the methyl esters with water and heptane, the samples were dried under N_2 and resuspended in 25 μl of carbon disulfide, and 1 μl was injected into a model 5890 gas chromatograph (Hewlett-Packard Co., Palo Alto, CA) for separation of fatty acid methyl esters. Fatty acids were identified by comparison of the relative retention times with fatty acid methyl ester standards.

PG formation. To determine PGE_2 production after agonist stimulation, the maintenance medium was removed, and the cells were washed twice with DPBS containing 0.1 μM BSA and then incubated for 20 min in 0.5 ml DPBS containing 0.1 μM BSA and 7.5 μM arachidonic acid. The buffer solution was removed and analyzed for PGE_2 production by RIA (15). Measurement of PGE_2 production by normal, infantile Refsum's disease, and Zellweger human skin fibroblasts was accomplished by mixing 100 μl of either standards or samples with 50 μl of anti-PGE_2 antibody (Anti-PGE_2 antibody, Advanced Magnetics Corp., Cambridge, MA). The mixture was incubated at 25°C for 30 min, 50 μl of [^{3}H]PGE_2 [10–15 × 10^3 dpm] was added, and the incubation was continued for 16 h at 4°C. Dextran-coated charcoal (400 μl) was added and after mixing and centrifugation at 3,000 *g* for 15 min, the radioactivity in 400 μl of the supernatant fluid was measured in a liquid scintillation spectrometer. Assay detection limits were 5 pmol of PGE_2, and 50% inhibition was obtained with 15 pmol/ml PGE_2. The assay has negligibly small amounts of cross reactivity with prostaglandins other than PGE_2.

To determine if soluble factor(s) produced by either the normal or Zellweger fibroblasts could affect PGE_2 production, conditioned medium from one fibroblast cell line grown for 48 h to 80% confluency was transferred to the opposite cell line. After 4 h of incubation, the conditioned medium was removed, the cells were washed with buffer and then exposed to 7.5 μM arachidonic acid for 20 min, and the medium was assayed for PGE_2 by RIA.

To determine if plasmalogen deficiency was responsible for the reduced PGE_2 production by the peroxisomal-deficient fibroblasts, confluent monolayers were incubated with modified Eagle's MEM containing 0.1 μM BSA supplemented with 5–12.5 μM 1-O-alkyl hexadecaglycerol (Sigma Chemical Co., St. Louis, MO) for 48 h. The medium was removed, and the cells were washed with buffer and then incubated for 20 min in 0.5 ml DPBS/0.1 μM BSA containing 7.5 μM arachidonic acid. The medium was removed and assayed for PGE_2 by RIA.

Modification of cellular peroxide tone. To determine whether the oxyradical scavenger catalase had any effect on PGE_2 formation, we incubated confluent cultures of normal or peroxisomal-deficient human skin fibroblasts with modified Eagle's MEM containing 0.1 μM BSA and 10–100 U/ml of catalase (46,500 U/mg protein, Sigma Chemical Co.) for 10 min to 2 h. After removal of the catalase-supplemented medium, the cells were washed with DPBS containing 0.1 μM BSA and then incubated for 20 min in 0.5 ml of DPBS/0.1 μM BSA supplemented with 7.5 μM arachidonic acid. The medium was removed and analyzed for PGE_2 by RIA.

To increase the half-life and intracellular permeability of the added catalase, we also tested catalase conjugated to polyethylene glycol (16). Normal and peroxisomal-deficient human fibroblasts were incubated in modified Eagle's MEM containing 1% heat-inactivated fetal bovine serum supplemented with 0.125, 0.25, and 0.5 mg/ml catalase-polyethylene glycol. After 24 h, the medium was removed, and the cells were washed and analyzed for PGE_2 production by RIA.

To determine if H_2O_2 had any effect on fibroblast PGE_2 production, confluent cultures of normal and peroxisomal-deficient human skin fibroblasts were incubated with modified Eagle's MEM containing 0.1 μM BSA supplemented with 1–100 μM H_2O_2. After 10 min, the H_2O_2 containing medium was removed and the cells were washed and analyzed for PGE_2 production by RIA.

Phorbol ester–stimulated PGE_2 formation. To determine whether PMA (LC Services Corp., Woburn, MA) was capable of increasing PGE_2 formation, we incubated monolayers of normal and Zellweger fibroblasts in Eagle's MEM containing 5% FBS supplemented with 10 nM PMA for 1–12 h. The PMA supplemented medium was removed and the cells were washed with buffer and then exposed to DPBS/0.1 μM BSA containing 7.5 μM arachidonic acid. After 20 min, the buffer solution was removed and the PGE_2 content was measured by RIA.

To determine whether the increase in PGE_2 formation after PMA was secondary to increased arachidonic acid release via enhanced phospholipase A_2 activity, normal and Zellweger fibroblasts were labeled with [^{3}H]arachidonic acid (0.5 μCi/ml) for 12 h in Eagle's MEM containing 5% FBS. After the medium was removed and the cells were washed, aliquots of the medium were counted in a liquid scintillation spectrometer to determine the amount of radioactivity taken up during the labelling period. Eagle's MEM containing 5% FBS supplemented with either 10 nM PMA or its vehicle was then added to the cultures for 12 h. At the appropriate time, 0.5 ml of medium containing the calcium ionophore A23187 (final concentration 2 μM) was added to the cultures. After 20 min, the remaining medium was extracted with ethyl acetate and the extract was resuspended in acetonitrile. Aliquots were removed and counted in the liquid scintillation spectrometer to determine the amount of lipid-soluble radioactivity that was released.

To examine whether inhibitors of transcription or translation would interfere with the effect of PMA on PGE_2 production, normal and Zellweger fibroblasts were incubated for 6 h in Eagle's MEM containing 5% FBS with or without 10 nM PMA. Additional incubations for 6 h contained PMA and either 1 μg/ml cycloheximide or 2 μg/ml actinomycin D (both from Sigma Chemical Co.). Cultures not exposed to PMA for 6 h but containing cyclohexamide or actinomycin D were also tested. After this medium was removed, the cells were incubated for 20 min with 7.5 μM arachidonic acid, and the medium was analyzed for PGE_2 by RIA.

To determine whether the effect of PMA on PGE_2 production was mediated through protein kinase C, normal and Zellweger fibroblasts were incubated for 6 h with or without PMA as indicated above. Additional incubations for 6 h contained PMA and either 100 μM H-7 [1-(5-isoquinoline sulfonyl)-2-methyl piperazine dihydrochloride, Seikagaku America, St. Petersburg FL] or 10 mM staurosporine (Sigma Chemical Co.). Cultures not exposed to PMA but containing H-7 or staurosporine were also tested. The cells then were incubated with 7.5 μM arachidonic acid and the medium analyzed for PGE_2 by RIA.

HPLC. The total radioactivity contained in a sample was determined initially by adding 4 ml of Budget Solve scintillation solution (Research Products International Corp., Mount Prospect, IL) to a 25-μl aliquot of the sample and counting in a model LS7000 liquid scintillation spectrometer (Beckman Instruments, Inc., Palo Alto, CA).

1. *Abbreviations used in this paper:* DPBS, Dulbecco's PBS; H-7, [1-(5-ioquinolinesulfonyl)-2-methylpiperazine dihydrochloride]; TBS, Tris-buffered saline.

Quenching was monitored with a ^{226}Ra external standard. Additional aliquots of the sample containing 10,000–15,000 dpm were transferred to siliconized glass vials, dried under a stream of nitrogen, and resuspended in 30 μl of HPLC-grade acetonitrile and 70 μl of acidified H_2O. The lipids were separated on a model 332 chromatograph (Beckman Instruments, Inc.) containing a 4.6 × 250-mm column (VYDAC, Hisperia, CA) with C_{18} reverse-phase 5 μm spherical packing. The solvent mixture consisted of water adjusted to pH 3.4 with phosphoric acid and an increasing acetonitrile gradient from 30% to 100% over 65 min. Radioactivity was detected by passing the column effluent mixed with Budget Solve scintillation solution through a HPLC radioactivity flow detector (Radiomatic Flo-One Beta, Canberra Corp., Meriden CT) and analyzed using software provided by Radiomatic.

Protein immunoblotting. Protein immunoblotting was carried out according to the method of Towbin with slight modification (17). Confluent cultures of normal, infantile Refsum's disease, and Zellweger human skin fibroblasts were grown in tissue culture flasks with 75 cm^2 surface area, washed, scraped twice in 3 ml of cold PBS, and placed on ice. The cells were sedimented at 4°C at 3,000 *g* for 10 min. After the supernatant fluid was removed, the cell pellet was lysed in 1 ml of PBS containing 1% Triton X-100 (Surfact Amps; Pierce Chemical Co., Rockford, IL), 10 μg/ml leupeptin, 1 mM phenylmethylsulfonylfluoride, 1 mM EDTA, and 1 mM diethyldithiocarbamic acid (all from Sigma Chemical Co.). The lysate was transferred to an Eppendorf tube and immersed in boiling water for 3 min, and then sedimented in a refrigerated microfuge at 27,000 *g* for 10 min. The supernatant solution was removed, placed in a Centricon-30 microconcentrator (Amicon Division, W. R. Grace and Co., Danvers, MA) and concentrated by centrifugation for 30-min intervals at 5,000 *g* and 4°C with 34° angle rotor. After the supernatant solution was concentrated to 100 μl, 20 μl was removed and assayed for protein content (18). The remainder was kept at 4°C and assayed within 16 h.

Proteins in the concentrated cell lysates containing the solublized PGH synthase were separated through 12.5% SDS-PAGE according to the method of Laemmli (19) using the Mini Protean gel system (Bio-Rad Laboratories, Richmond, CA). Sample aliquots equivalent to 100 μg of protein per lane were denatured by heating to 96°C for 4 min in an SDS dissociation buffer containing β-mercaptoethanol. To confirm separation by SDS-PAGE, a standard mixture of 7 proteins was included with each gel (High Range Molecular Weight Standard Mixture, Bethesda Research Laboratory, Bethesda, MD). Proteins separated by SDS-PAGE were electrophoretically transferred at 4°C to 0.45 μm nitrocellulose paper (BA 85, Midwest Scientific, St. Louis, MO). Gels were stained with Coomassie Brilliant Blue to confirm that the transfer efficiency was > 90% and that all samples were transferred uniformly. Nitrocellulose membranes were saturated overnight at 4°C in Tris-buffered saline (TBS) containing 10% FBS and 2% BSA.

After saturation, the nitrocellulose membrane was washed in TBS/0.05% Tween and once in TBS for a total of 30 min. The membrane was incubated for 4 h at 25°C in 7.5 ml of TBS/2% BSA, pH 8.0, containing rabbit anti-PGH synthase antibody (Polyclonal rabbit anti-PGH synthase, Oxford Biomedical Research, Oxford, MI) diluted 1:100. The nitrocellulose membrane then was washed as before and incubated for 2 h at 25°C in 7.5 ml of TBS/2% BSA, pH 8.0, containing goat anti-Rabbit IgG conjugated with alkaline phosphatase (Sigma Chemical Co.) diluted 1:10,000. After washing, the nitrocellulose was treated with 5-bromo-4-chloro-3-indoyl phosphate and nitroblue tetrazolium (both from Sigma Chemical Co.) for color development and visualization.

mRNA analysis. Total RNA was isolated from cell cultures under basal conditions and after treatment with PMA for 2–16 h by extraction with guanidium isothiocyanate and subsequent cesium chloride centrifugation according to the methods described by Chirgwin et al. (20) and Glisin et al. (21). 15 μg of total RNA was fractionated on 1.5% agarose/formaldehyde gels and transferred to Genescreen Plus (NEN Research Products, Boston, MA) hybridization membrane by capillary blotting. The RNA was cross-linked to the membrane by exposure to ultraviolet light.

The membranes were hybridized to a 2.5-kb cDNA probe for human PGH synthase (Oxford Biomedical Research) after random prime labeling with [^{32}P]cytidine 5′-triphosphate (dCTP) according to methods outlined by Sambrook et al. (22). The membranes were washed twice in 0.3 M sodium chloride and 0.03 M sodium citrate at room temperature and then exposed to Kodak XAR-5 film at −80°C with intensifying screens. To determine the amount of RNA loaded on each lane, the membrane also was probed with radiolabeled β-actin cDNA kindly supplied by Dr. Gary Hunninghake, University of Iowa College of Medicine.

Reagents. [1-^{14}C]arachidonic acid (58.4 mCi/mmol), [5,6,8,9,11,12,14,15-^{3}H]-arachidonic acid (110 Ci/mmol), and [5,6,8,11,12,14,15 (N)-^{3}H]-PGE_2 (160 Ci/mmol) were purchased from Amersham Corp. (Arlington Heights, IL). Deoxycytidine 5′-triphosphate tetra(triethylammonium) salt [α-^{32}P], 3,000 Ci/mmol, was purchased from Dupont NEN Research Products. Arachidonic acid was purchased from Nu-Check Prep (Elysian, MN). All organic solvents, either reagent or HPLC grade, were purchased from Fisher Scientific Co. (Fair Lawn, NJ).

Statistics. Statistical analysis was performed using a one-way ANOVA. Statistical significance was determined at the $P < 0.05$ level and was tested according to the method of Scheffé (23).

Results

PG formation. Confluent monolayers of fibroblasts were incubated with 7.5 μM arachidonic acid for 20 min and the medium was assayed for PGE_2 by RIA. Fig. 1 demonstrates a substantial reduction in PGE_2 release by both the Zellweger and infantile Refsum's disease human skin fibroblasts when compared to normal human fibroblasts. To determine whether the PGE_2 reduction may be due to a lesser ability of the mutant cells to channel arachidonic acid into PGs, normal and Zellweger human skin fibroblasts were incubated with increasing concentrations of arachidonic acid for 20 min, and the medium was assayed for PGE_2 by RIA. Fig. 2 demonstrates that while some increase in PGE_2 production occurred in the Zellweger cells, the defect was not overcome by increasing the availability of arachidonic acid to the cells. At all concentrations tested, the Zellweger fibroblasts produced substantially less PGE_2 than the normal cells. Longer periods of incubation with arachidonic acid also failed to correct the defect in Zellweger fibroblast PGE_2 production. When the cells were incubated with 7.5 μM arachidonic acid for 90 min and the medium was assayed for PGE_2 by RIA, PGE_2 formation in Zellweger fibro-

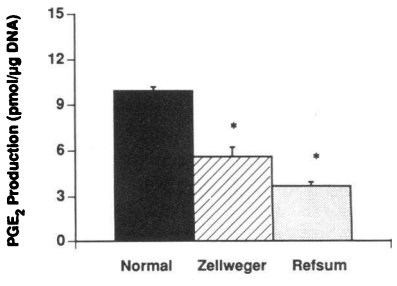

Figure 1. Arachidonic acid–stimulated PGE_2 formation in normal, Zellweger, and infantile Refsum's disease fibroblasts. The cells were incubated for 20 min with 0.5 ml of DPBS containing 0.1 μM BSA and 7.5 μM arachidonic acid. The PGE_2 content of the medium was measured by RIA. The bars represent the mean±SEM of 8 separate determinations in normals (three different experimental days, two in triplicate, one in duplicate), 11 in Zellweger's (four different experimental days, three in triplicate and one in duplicate), and 3 in infantile Refsum's cells (one experiment, data in triplicate). *$P < 0.05$ in Zellweger and Refsum's vs. normal fibroblasts.

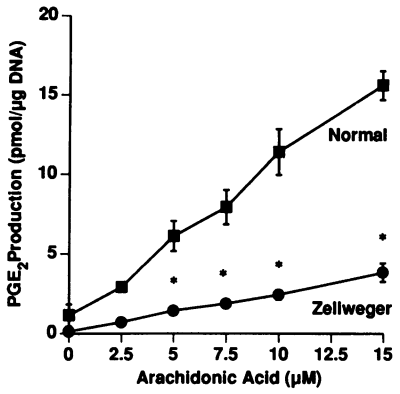

Figure 2. Effect of arachidonic acid concentration on PGE_2 production in normal and Zellweger fibroblasts. The cells were incubated for 20 min in DPBS containing 0.1 μM BSA and 2.5–15 μM arachidonic acid. The PGE_2 content of the medium was measured by RIA. Error bars represent the SEM for three separate determinations. $*P < 0.05$ in Zellweger vs. normal fibroblasts.

blasts remained significantly less than the normal fibroblasts (20.5±1.04 vs. 30.6±4.5 pmol/μg DNA, $P < 0.05$).

Analysis of the cellular fatty acid composition by gas chromatography indicated no difference in arachidonic acid content between normal (8.4% of total cell fatty acids) Zellweger (8.4%) and infantile Refsum's disease (8.1%) fibroblasts. However, Table I demonstrates that the reduction in PGE_2 formation also occurs in the Zellweger cells in response to A23187, where the arachidonic acid is derived from intracellular stores. Prior supplementation with small amounts of arachidonic acid failed to correct the decrease in A23187-stimulated PGE_2 production in the Zellweger cells.

To determine whether arachidonic acid was being shunted to other metabolic pathways, control and peroxisomal-deficient fibroblasts were incubated with 7.5 μM [1-^{14}C]-arachidonic acid for 20 min and the radioactivity contained in the medium assayed by HPLC. Normal human skin fibroblasts converted 4.7% of the radioactivity to a single component with a HPLC retention time of 16.5 min, identical to that of standard PGE_2 (Fig. 3, *top*). Both the Zellweger and infantile Refsum's disease fibroblasts converted less [1-^{14}C]arachidonic acid to PGE_2, and no other cyclooxygenase or lipoxygenase products were detected (Fig. 3, *middle* and *bottom*, respectively). Incubation of [1-^{14}C]arachidonic acid–labeled peroxisomal-deficient cells with agonists such as calcium ionophore A23187, bradykinin, histamine, or serotonin also failed to stimulate release of any alternative radioactive cyclooxygenase or lipoxygenase products (data not shown).

Conditioned medium and alkyl glycerol supplementation. Another possibility is that the peroxisomal-deficient cells fail to produce a necessary activator, or produce an inhibitor of PG formation. To evaluate this, a crossover experiment was done with conditioned medium. After at least 48 h of growth, the conditioned medium from one fibroblast line was immediately

Table I. PGE_2 Production

Skin fibroblasts	Arachidonic acid added	PGE_2 formed
	μM	*pmol/ml*
Normal	0	32.7±1.6
Zellweger	0	4.9±1.3*
Zellweger	1	4.3±2.5*
Zellweger	2	2.8±0.1*

Normal and Zellweger cells were grown to 90% confluency. Where indicated, the cultures were supplemented with 1 or 2 μM arachidonic acid for 1 h. After the medium was removed, the cells were exposed to 2 μM A23187 for 20 min and the buffer solution was assayed for PGE_2 production by RIA. The data represent the mean±SEM of three separate determinations. $*P < 0.05$. Normal vs. Zellweger cells.

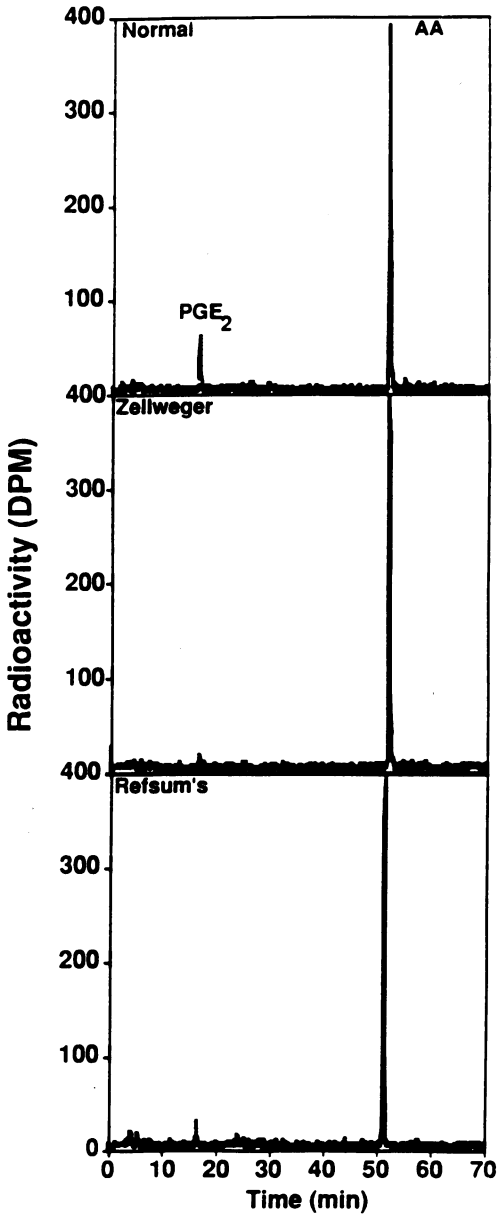

Figure 3. HPLC profile of radioactive metabolites present in the medium after incubation of fibroblasts with radioactive arachidonic acid. The cells were incubated with 7.5 μM [1-^{14}C]arachidonic acid in serum-free MEM containing 0.1 μM BSA for 20 min. The medium was removed, acidified, extracted with ethyl acetate, dried under N_2, and resuspended in acetonitrile. An aliquot containing at least 10,000 dpm was separated by reverse-phase HPLC. Radioactivity was assayed with an on-line, flow through scintillation counter. In the normal fibroblasts, 4.7% of the radioactivity eluted at 16.4 min, identical to the retention time of a PGE_2 standard. Arachidonic acid has a retention time in this system of 50.4 min.

transferred to the opposite cell line. Conditioned medium from the Zellweger fibroblasts did not inhibit normal fibroblast PGE_2 production. After 4 h in Zellweger-conditioned medium, PGE_2 production by the normal fibroblasts increased from a control value of 6.36±0.06 to 9.73±0.06 pmol/μg DNA. Likewise, conditioned medium from normal fibroblasts did not correct the attenuated PGE_2 production in Zellweger fibroblasts. After 4 h in conditioned medium from normal cells, PGE_2 production by the Zellweger cells was 3.74±0.05 pmol/μg DNA, still significantly reduced as compared with the amount released by normal fibroblasts. Thus, the deficiency in the Zellweger fibroblast is not due to either the production of a soluble, transferrable inhibitor, or failure to produce a transferrable activator.

An alternate possibility is that by virtue of the plasmalogen deficiency that occurs in peroxisomal-deficient fibroblasts, an important potential intracellular source of arachidonic acid for prostaglandin formation is reduced. To test this possibility, subconfluent monolayers of Zellweger fibroblasts were incubated for 48 h in medium supplemented with 1-O-alkyl hexadecaglycerol. Exposure to this plasmalogen precursor did not appreciably increase arachidonic acid–stimulated PGE_2 production in the Zellweger fibroblasts. PGE_2 production by cells not exposed to this compound was 1.42±0.06 pmol/μg DNA, compared with 1.48±0.01 and 1.86±0.19 pmol/μg DNA in cells exposed to 7.5 and 12.5 μM 1-O-alkyl hexadecaglycerol, respectively.

Alteration of peroxide tone. Because of the peroxisomal deficiency, it is possible that H_2O_2 is not properly metabolized in the Zellweger cells. For example, H_2O_2 may build up because of the aberrant localization of catalase within the cells (24), and thereby inactivate PGH synthase. To determine if elevated peroxide content may be a factor in inhibiting PGE_2 production, 10–100 μM catalase was incubated with the Zellweger cells for 60–120 min, and the cells then were exposed to 7.5 μM arachidonic acid for 20 min. RIA analysis revealed that this pretreatment with catalase did not increase Zellweger fibroblast PGE_2 production (data not shown). Because catalase was added to the medium, it may have had limited access to the intracellular space. Therefore, we attempted to more effectively increase the intracellular concentration of catalase by preincubating the cells for 24 h with 0.0125, 0.25, and 0.5 mg/ml catalase conjugated to polyethylene glycol. Previous work by Beckman et al. (16) suggests that this is a more efficient way of increasing the cellular catalase content and thereby lower intracellular H_2O_2 concentration. However, exposure to the catalase-polyethylene glycol preparation also did not increase PGE_2 production when the Zellweger fibroblasts were subsequently incubated with 7.5 μM arachidonic acid for 20 min (data not shown).

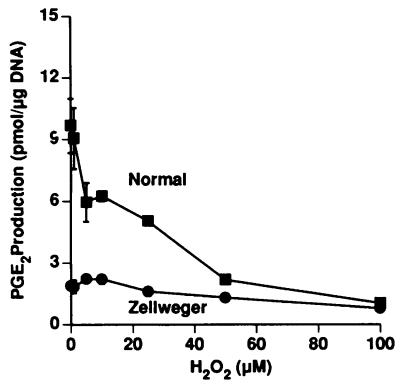

Figure 4. Effect of increasing concentrations of H_2O_2 on normal and Zellweger fibroblast PGE_2 production. The fibroblasts were incubated for 10 min with modified MEM containing 0.1 μM BSA and 5–100 μM H_2O_2. After removal of the medium and washing, the cells were incubated with 7.5 μM arachidonic acid for 20 min and the medium assayed for PGE_2 by RIA. Data points represent the mean±SEM for three separate determinations.

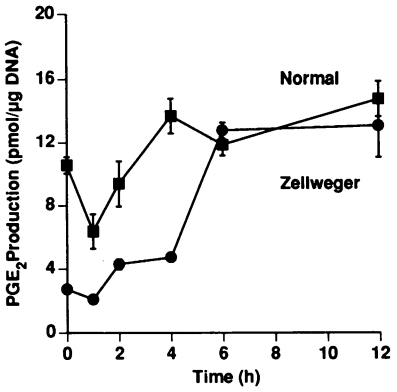

Figure 5. Effect of increasing time of exposure to PMA on PGE_2 production by normal and Zellweger fibroblasts. The fibroblasts were incubated for 1–12 h with MEM containing 5% heat-inactivated FBS supplemented with 10 nM PMA. At the appropriate time, the medium was removed and the cells were washed and exposed for 20 min to DPBS containing 0.1 μM BSA and 7.5 μM arachidonic acid. PGE_2 was measured by RIA, and each point represents the mean±SEM for three separate determinations.

The alternative possibility is that because of aberrant catalase localization, the peroxide tone of the cell may be too low to trigger PG formation. If so, addition of H_2O_2 may overcome this deficiency. To test this, we incubated confluent monolayers of normal and Zellweger human skin fibroblasts with 1–100 μM H_2O_2 for 10 min, removed this medium, and then exposed the cells to 7.5 μM arachidonic for 20 min. Fig. 4 demonstrates that PGE_2 formation in the Zellweger fibroblasts was not increased by prior treatment with H_2O_2. Furthermore, incubation of the normal fibroblasts with H_2O_2 resulted in a dose-dependent reduction in PGE_2 formation. After exposure to 50 or 100 μM H_2O_2, the amount of PGE_2 produced by the normal cells was reduced to the level observed with the Zellweger cells.

Effect of phorbol ester. PMA has been shown to increase PG synthesis in many cells by increasing PGH synthase formation (25–27). To determine if this would restore the defect in PGE_2 production, monolayers of normal and Zellweger fibroblasts were incubated with 10 nM PMA for 1–12 h. After this medium was removed and the cells washed, they were incubated with DPBS/0.1 μM BSA supplemented with 7.5 μM arachidonic acid and the medium assayed by RIA. Fig. 5 demonstrates that after a 6- or 12-h incubation with PMA, PGE_2 formation in the Zellweger fibroblasts was restored to a level identical to that seen in the normal human skin fibroblast treated in the same manner.

PMA's effect to increase PGE_2 formation might be through its previously described effect of increasing phospholipase A_2 activity and, hence, increasing the availability of arachidonic acid (28). To determine whether this might be the mechanism of the PMA effect, monolayers of normal and Zellweger fibroblasts were labelled with [^{3}H]arachidonic acid for 12 h. This medium was removed and the cells were incubated with 5% FBS supplemented with 10 nM PMA for an additional 12 h. A23187 at a final concentration of 2 μM was added to the cultures for 20 min, and the release of arachidonic acid was determined. Table II indicates that treatment with PMA in both the normal and Zellweger fibroblasts failed to augment the release of radioactivity from the pulse-labeled cells.

Table II. Release of Arachidonic Acid Radioactivity

Skin fibroblasts	Radioactivity released	
	Buffer	PMA
Normal	5.2±0.4	5.6±0.4
Zellweger	5.3±0.3	4.8±0.4

Normal and Zellweger fibroblasts were grown to confluency and labeled with [^{3}H]arachidonic acid for 12 h. After removing the radiolabeled medium and counting aliquots to determine uptake, the fibroblasts were incubated in medium with or without 10 nM PMA for an additional 12 h, and 2 μM A23187 was then added to the cultures for 20 min. The medium was extracted with ethyl acetate, and aliquots were counted in a liquid scintillation spectrometer to determine the release of radioactivity. This was calculated as the quotient of the counts released into the ethyl acetate extract of the medium, divided by the counts incorporated in the cells at the end of the pulse-labeling period. The data represent the mean±SEM of three separate determinations made with cultures studied on the same day.

To further investigate the mechanism through which PMA treatment increases PGE_2 production in the peroxisomal deficient fibroblasts, we included either 1 μg/ml cycloheximide or 2 μg/ml actinomycin D with 10 nM PMA during the initial 6-h incubation. The cells then were exposed to 7.5 μM arachidonic acid for 20 min. Fig. 6 demonstrates that in the normal fibroblasts (*top*), treatment with PMA increased PGE_2 production about twofold. Inclusion of either actinomycin D or cycloheximide eliminated the stimulatory effect of PMA (*top, right*), and these inhibitors also reduced arachidonic acid stimulated PGE_2 production in the absence of PMA pretreatment (*top, left*). In the Zellweger fibroblasts (*bottom*), PMA also substantially increased PGE_2 production. As was observed in the normal fibroblasts, inclusion of either cycloheximide or actinomycin D reduced basal PGE_2 production and almost totally eliminated the PMA mediated increase.

To determine whether PMA produced this response by activating protein kinase C, we included 100 μM H-7 or 10 nM staurosporine with 10 nM PMA during an initial 6 h incubation and the cells then were exposed to 7.5 μM arachidonic acid for 20 min. Fig. 7 demonstrates that inclusion of H-7 and staurosporine reduced PMA stimulated PGE_2 production by the normal fibroblasts, with H-7 being somewhat more effective (*top, right*). Staurosporine and H-7 also reduced arachidonic acid stimulated PGE_2 production in the absence of PMA stimulation (*top, left*). Likewise, in the Zellweger fibroblasts (*bottom*), staurosporine and H-7 were effective in reducing both PMA-stimulated and basal PGE_2 production, with H-7 again being more effective.

PGH synthase protein. We sought to determine whether the decrease in PGE_2 production might be secondary to a difference in PGH synthase in the peroxisomal deficient fibroblasts. Protein immunoblotting was carried out on cell lysates from normal, Zellweger, and infantile Refsum's disease fibroblasts. Fig. 8 is a representative immunoblot demonstrating the presence of a single 70-kD band in the lane containing the ram seminal vesicle PGH synthase standard. A 72–74-kD doublet was observed when all three human skin fibroblast cell line extracts were blotted with this antibody. A similar doublet has been previously demonstrated with anti-PGH synthase in $p60^{V\text{-}SRC}$ transformed 3T3 fibroblasts (29).

PGH synthase mRNA. To determine whether a difference in PGH synthase mRNA could account for our PGE_2 production findings, we carried out mRNA analysis in normal and Zellweger human skin fibroblasts. Fig. 9 is representative of three different Northern blots performed on RNA extracted from resting, unstimulated cultures. A single 4.2-kb band is visible in the normal skin fibroblasts (lane *1*); this band is not present in the peroxisomal-deficient Zellweger fibroblasts. We also probed these Northern blots with β-actin cDNA and detected a band in both the normal and peroxisomal-deficient cell extracts. Thus, the absence of PGH synthase mRNA in the unstimulated Zellweger fibroblasts is not due to degradation of the mRNA in the sample.

The PGH synthase mRNA response of normal human skin fibroblasts between 2 and 16 h after PMA stimulation is shown in Fig. 10, lanes *1–4*. Two PGH synthase mRNA bands were detected. The 4.2-kb mRNA transcript, which had been detected in the quiescent cells (Fig. 9), increased over time up to 8 h and then subsequently decreased. Densitometric analysis indicated that the maximum increase which occurred at 8 h was twofold. A smaller transcript which migrated with a 2.8-kb rRNA standard also was detected. This 2.8-kb mRNA ap-

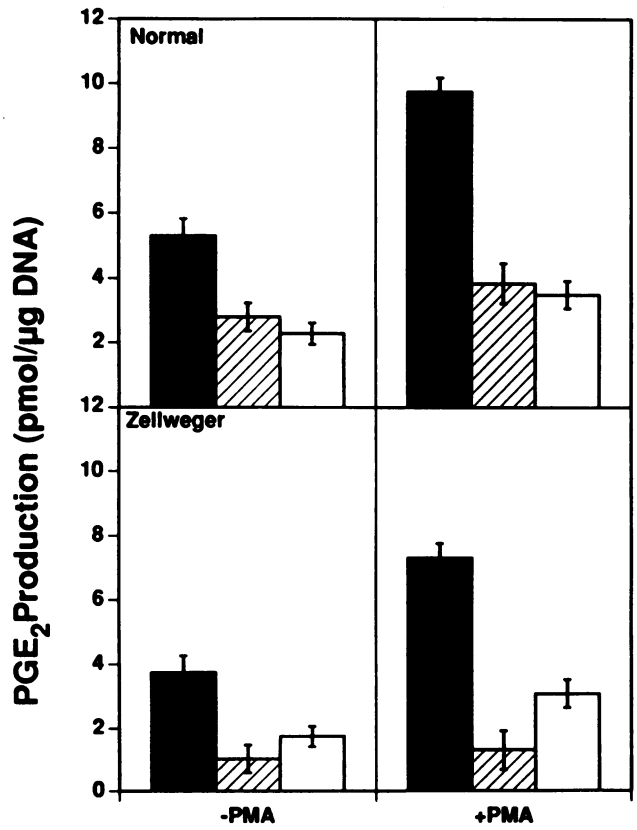

Figure 6. Effect of cycloheximide and actinomycin D on PMA-stimulated PGE_2 production in normal and Zellweger fibroblasts. Normal (*top*) and Zellweger (*bottom*) fibroblasts were incubated for 6 h with modified MEM containing 5% FBS with or without 10 nM PMA; (■) control. Additional incubations with or without PMA contained either (▨) 2 μg/ml actinomycin D or (□) 1 μg/ml cyclohexamide. At the appropriate time, the medium was removed and the cells were washed and exposed to 7.5 μM arachidonic acid for 20 min. PGE_2 was measured by RIA, and the bars represent the mean±SEM for three separate determinations.

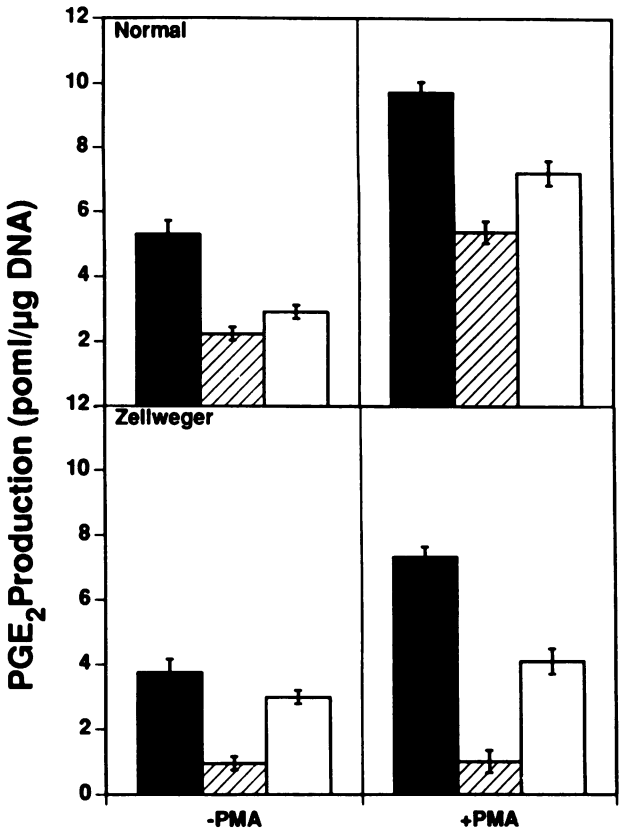

Figure 7. Effect of protein kinase C inhibitors on PMA-stimulated PGE_2 production in normal (*top*) and Zellweger (*bottom*) fibroblasts. The cells were incubated for 6 h with modified MEM or 5% FBS with or without 10 nM PMA; (■) Additional incubations with or without PMA contained either (□) 100 µM H-7 or (□) 10 nM staurosporine. At the appropriate times, the medium was removed and the cells were washed and exposed to 7.5 µM arachidonic acid for 20 min. PGE_2 was measured by RIA, and each bar represents the mean±SEM for three separate determinations.

peared in parallel with the increased expression of the 4.2-kb mRNA transcript in the normal skin fibroblasts, and densitometric analysis indicated that it reached the same twofold maximum increase (Fig. 10, lanes *1–4*).

In peroxisomal-deficient Zellweger fibroblasts, the 4.2-kb mRNA transcript was detected at 2 h after PMA stimulation (Fig. 10, lanes *5–8*), and the maximum increase in the 4.2-kb mRNA transcript observed by densitometric tracing occurred between 6 and 8 h (Fig. 10, lanes *6* and *7*). However, the 2.8-kb mRNA transcript was not detected at any of these times after PMA stimulation in the peroxisomal-deficient cells.

Discussion

These results demonstrate that peroxisomal-deficient human skin fibroblasts have a marked decrease in both arachidonic acid and calcium ionophore-stimulated PG production. The substantial variability in the absolute values presented in Figs. 2, 4, and 5 probably is due to differences in passage number and confluency, factors that could not be easily controlled because of the different properties of the various cell cultures. However, despite this quantitative variability, PGE_2 production by peroxisomal-deficient human skin fibroblasts was always reduced when compared with that of normal skin fibroblasts under the experimental conditions utilized.

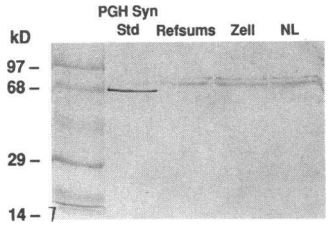

Figure 8. Protein immunoblot analysis of PGH synthase. Normal, infantile Refsum's disease and Zellweger human skin fibroblasts were grown to confluency. The medium was removed, and the cells were scraped in ice-cold PBS and lysed in 3 ml of PBS containing 1% Triton X-100, 10 µg/ml leupeptin, 1 mM EDTA, 1 mM diethyldithiocarbamic acid, and 1 mM phenylmethylsulfonylfluoride. The lysate was boiled and the supernatant concentrated to 100 µl in Centricon-30 filters. 75 µg of cell protein was loaded onto 12.5% SDS denaturing gels and electrophoresed for 2 h. The proteins were transferred to nitrocellulose. The nitrocellulose was then saturated at 4°C in TBS supplemented with 10% FBS and 2% BSA. The next day, 7.5 ml of TBS/10% FBS/2% BSA containing a 1:100 dilution of sheep anti-PGH synthase was added for 4 h. The primary antibody was removed and 7.5 ml of TBS/10% FBS/2% BSA, containing a 1:10,000 dilution of anti-rabbit IgG conjugated with alkaline phosphatase was added for an additional 2 h. The blots were stained with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium and developed for 15 min. Standard PGH synthase (lane *2*) developed into a single band at 69 kD, and bands migrating nearly identical to this standard were detected in each of the human skin fibroblast lysates. Abbreviations: PGH Syn Std, PGH synthase standard; NL, normal human skin fibroblasts; Refsums, Refsum's disease fibroblasts; Zell, Zellweger disease fibroblasts.

Peroxisomes are the site of PG catabolism through β-oxidation (30), but this organelle is not considered to be involved in PG production. PGH synthase, the rate-limiting enzyme in PG synthesis (12), is present in the endoplasmic reticulum and nuclear membrane (31), and there is no indication that the other enzymes in the synthetic pathway are located in the peroxisomes. We also are unaware of any clinical reports of decreased PG production in peroxisomal deficiency diseases. Therefore, the observation that PG formation is reduced in peroxisomal-deficient cells is completely unexpected.

We initially explored whether the defect might involve arachidonic acid availability or metabolism. No decrease was observed, however, in the arachidonic acid content of the Zellweger cell lipids. This is consistent with our previous finding that the uptake and distribution of radioactive arachidonic acid in Zellweger cells is the same as in normal human skin fibro-

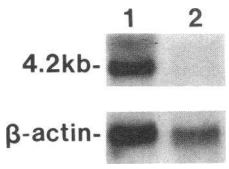

Figure 9. PGH synthase mRNA analysis in unstimulated fibroblasts. Normal and Zellweger human skin fibroblast mRNA was prepared by guanidium isothiocyanate extraction and cesium chloride centrifugation. RNA was fractionated on 1.5% agarose/formaldehyde gels, transferred to Genescreen Plus, and cross-linked by ultraviolet light to the membrane. The membranes were hybridized to a 2.5-kb cDNA probe after random prime labeling with [α-^{32}P] dCTP. After several washes with a solution containing 0.3 M NaCl and 0.03 M sodium citrate, the membranes were exposed to Kodak XAR-5 film at −80°C with intensifying screens. Each membrane also was probed with a β-actin cDNA. Lane *1* (normal fibroblast) hybridizes at 4.2 kb. In lane *2* (Zellweger fibroblast), there is no detectable PGH synthase mRNA.

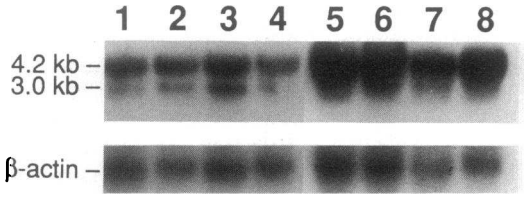

Figure 10. PGH synthase mRNA analysis in PMA stimulated fibroblasts. Normal and Zellweger human skin fibroblasts were treated with 10 nM PMA for 2–16 h and then stimulated with 7.5 μM arachidonic acid for 20 min as described in Fig. 5. Normal and Zellweger human skin fibroblast mRNA was subsequently prepared as described in Fig. 9. Lanes *1–4* are normal human skin fibroblasts treated with PMA for 2, 6, 8, and 16 h, respectively. Lanes *5–8* are Zellweger fibroblasts treated with PMA for 4, 6, 8, and 16 h, respectively. Both a 3.0- and 4.2-kb transcript are detected in the normal fibroblast whereas only the 4.2-kb transcript is detected in the Zellweger fibroblast.

blasts (8). Furthermore, exposure of the Zellweger fibroblasts to elevated amounts of arachidonic acid failed to correct the deficiency, and HPLC analysis demonstrated that arachidonic acid is not being shunted into other eicosanoid products in the peroxisomal-deficient cells. The defect was also not overcome by the administration of *O*-alkylglycerol, which restores the ability of peroxisomal-deficient cells to synthesize plasmalogens (32). However, the possibility that the deficient cells do not convert the *O*-alkylglycerol precursor to a specifically required plasmologen, such as the 1-vinyl ether 2-arachidonyl species, cannot be ruled out by these results.

Since the reduction in agonist stimulated PGE_2 formation apparently is not related to arachidonic acid availability, it probably involves some aspect of the biosynthetic pathway. Western blots demonstrate that both the normal and mutant fibroblast lysates contain a 72–74-kD doublet that cross-reacts with an antibody to PGH synthase. This is consistent with the reported PGH synthase subunit molecular mass of about 72 kD (33). The presence of a doublet rather than a single component in the Western blots also was noted in the normal fibroblasts, and it has been reported in transformed 3T3 cells (29).

The results in Fig. 10, lanes *1–4,* demonstrate that under the stringency conditions selected for this study, our PGH synthase probe is able to detect two forms of PGH synthase mRNA. Between 2 and 16 h after PMA stimulation, 4.2- and 2.8-kb transcripts were observed in the normal fibroblast extracts. By contrast, only the 4.2 kb mRNA transcript was detected in the unstimulated normal human skin fibroblasts (Fig. 9, lane *1*). Studies with Swiss 3T3 cells suggest that the larger transcript encodes the inducible form of PGH synthase, called PGHS-2 or Cox-2, which is an immediate early response gene (34). The growth medium used for these studies contained 20% FBS. Apparently, this provided enough mitogenic activity to maintain the presence of some 4.2-kb PGH synthase mRNA in the unstimulated fibroblasts. In this regard, a 4.8-kb PGH synthase mRNA transcript has been detected in unstimulated normal human monocytes (35), and a 5.5-kb transcript is present in unstimulated normal human lung fibroblasts (36). In these cells, however, a 2.7–2.8-kb PGH synthase mRNA transcript also was detected (35, 36). Why the smaller mRNA transcript, which probably encodes the constitutive PGH synthase-1 or Cox-1 (34), was detected in the normal human skin fibroblasts only after exposure to PMA cannot be explained.

No PGH synthase mRNA was detected by Northern blot analysis in the unstimulated Zellweger fibroblasts even though the β-actin probe demonstrated that substantial quantities of mRNA were added to the gels (Fig. 9). Since these cells produce a small amount of PGE_2 and contain PGH synthase protein as determined by Western blotting (Fig. 8), PGH synthase mRNA transcripts must occur at some stage in the Zellweger cells. Either the unstimulated Zellweger cells were assayed at a stage when these transcripts are no longer retained, or the Northern analysis is too insensitive to detect the low levels that are present. The latter explanation seems more likely because Northern blot analysis is known to have low sensitivity in detecting transcripts for eicosanoid-producing enzymes in certain cells, e.g., 12-lipoxygenase mRNA expression in human erythroleukemia cells (37).

It is known that PGH synthase can be induced by treatment of cell cultures with phorbol esters (27, 38–40). This prompted us to investigate whether exposure of the peroxisomal-deficient human skin fibroblasts to PMA might overcome the deficit in PGE_2 production. A time-dependent increase in PGE_2 formation was observed (Fig. 5), and this was associated with the appearance of the 4.2-kb PGH synthase mRNA transcript (Fig. 10, lanes *5–8*). No clearly visible, distinct 2.8-kb transcript was detected in the Zellweger cells at any time after PMA exposure. When the normal fibroblasts were treated with PMA, the abundance of 4.2-kb transcript, which was present in these cells in the unstimulated state, also increased. In addition, a 2.8-kb transcript appeared in the normal cell extracts and increased in parallel with the larger transcript (Fig. 10, lanes *1–4*). This suggests that in the normal human fibroblasts, the 2.8-kb PGH synthase mRNA may be derived from the 4.2-kb transcript. In this regard, a parallel increase in the expression of spliced and unspliced PGH synthase transcripts has been observed when human lung fibroblasts are treated with PMA and serum (36). Alternatively, the 2.8-kb transcript may represent the inducible form of PGH synthase that was observed previously when human endothelial cells were exposed to interleukin 1 (41). The failure to observe any PMA-induced increase in total arachidonic acid release from either the normal or Zellweger fibroblast (Table II), an indirect measure of phospholipase A_2 activity, is consistent with the effect of PMA occurring at the level of PGH synthase in both cases.

After exposure to PMA, there was a 4-h delay before PGE_2 production reached maximum levels in the normal fibroblasts and a 6-h delay in the Zellweger cells (Fig. 5). Such a lag period also is compatible with a mechanism involving PGH synthase gene expression. Likewise, the inhibitory effects of actinomycin D and cycloheximide suggest that gene expression is involved. Results obtained in 3T3 fibroblasts exposed to platelet derived growth factor are consistent with this interpretation; the increase in PGE_2 synthesis resulting from production of PGH synthase exhibited a lag period of 2–6 h and was inhibited by cycloheximide and actinomycin D (42). Since PMA activates protein kinase C, this enzyme probably mediates the response in the human skin fibroblasts, as it does in other cell lines (12). This is supported by the fact that staurosporine and H-7 inhibited the increase in PGE_2 production resulting from exposure to PMA.

The alternative possibility that the activity of the PGH synthase is reduced in peroxisomal-deficient cells appears to be excluded by the present results. One factor that regulates PGH synthase activity is the intracellular peroxide tone (43). Peroxi-

somes generate H_2O_2 through fatty acid β-oxidation (3, 44) and they contain catalase, the enzyme that breaks down H_2O_2 (1, 2). Depending on which of these processes predominates, a peroxisomal deficiency might either increase or decrease the peroxide tone. However, addition of neither H_2O_2 nor a polyethylene glycol–catalase complex stimulated PGE_2 output by the Zellweger cultures. The concentrations of H_2O_2 tested apparently were effective because, as in endothelial cells (45), prostaglandin production was reduced when the normal fibroblasts were treated with these quantities of H_2O_2 (Fig. 4). Based on these findings, it seems unlikely that abnormalities in peroxide tone are responsible for the decreased PGE_2 formation in the peroxisomal deficient cells.

As opposed to the present findings, Tiffany et al. (46) have observed that PGE_2 production is increased in peroxisomal deficient fibroblasts. The culture conditions, however, were quite different in the two studies. Tiffany et al. (46) utilized a gas phase containing 10% CO_2 and supplemented the growth media with 10% FBS, whereas we used 5% CO_2 and 20% FBS. In addition, Tiffany et al. (46) tested the fibroblasts after exposure to a serum-free medium supplemented with 2% TCM, a proprietary mixture containing vitamins, growth factors, and fatty acids. Some or all of these factors might have modified the response in the mutant cells, leading to an increase rather than the decrease in PGE_2 formation that we observed.

In conclusion, our findings demonstrate that under commonly used culture conditions, the capacity of peroxisomal-deficient human skin fibroblasts to produce PGs is impaired. Therefore, peroxisome integrity appears to be necessary for normal PG formation, a relationship not previously recognized in eukaryotic cells. These results also suggest the possibility that some of the abnormalities associated with peroxisomal deficiency syndromes may be related to an inability to produce adequate amounts of PGs.

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