Modification of Cultured Madin–Darby Canine Kidney Cells with Dietary Unsaturated Fatty Acids and Regulation of Arachidonate Cascade Reaction

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Madin–Darby canine kidney (MDCK) cells were modified with dietary unsaturated fatty acids. The effects on the fatty acid composition in each phospholipid class and the formation of prostanoids upon stimulation were studied, from which the specificity of metabolism of individual unsaturated fatty acids and the regulation of arachidonate cascades in the modified cells were discussed. C18 unsaturated fatty acids were preferentially incorporated into phosphatidylincholine (PC) over phosphatidylethanolamine (PE), but arachidonic acid (20:4(n-6)) derived from γ-linolenic acid (18:3(n-6)) was much more predominant in PE than PC. The fatty acid level in PE ranged from about 26–28% when the cells were modified with 20:4(n-6) or 5,8,11,14,17-eicosapentaenoic acid (20:5(n-3)), indicating the limitation of the storage of the eicosapolyenoic acids. The extra amounts appeared to be stored in PC. 18:3(n-6) was comparable to 20:4(n-6) to raise the level of 20:4(n-6) in PE, but not in PC which had half of 20:4(n-6) in PE. The supplementation of linoleic acid (18:2(n-6)), 18:3(n-6), and 20:4(n-6) caused significant increases in the synthesis of prostaglandin (PG)E₂. Up to almost the same level when the modified cells were stimulated with 50 nm PMA and 100 nm A23187 for 24 h. The cultured cells modified with eicosapolyenoic acids including 20:3(n-6), 20:4(n-6), and 20:5(n-3) were found to be inhibitory for the induction of PGF₂α, synthetic activity involving de novo synthesis of PG endoperoxide synthase, suggesting negative feedback regulation of the modified cells.

Key words: prostanoid; arachidonate cascade; PG endoperoxide synthase; dietary unsaturated fatty acid; Madin–Darby canine kidney cells

Prostaglandins and related compounds called eicosanoids are synthesized from eicosapolyenoic acids of which the major precursor is 20:4(n-6). The 20:4(n-6) should be supplied from the dietary sources for mammals. When the 20:4(n-6) is incorporated into membrane phospholipids, it remains as their acyl moieties until stimulation with a variety of agonists. Once the activation is induced upon stimulation, 20:4(n-6) is provided as precursors of bioactive lipid mediators via the pathway called arachidonate cascades involving cyclooxygenases and lipooxygenases. In addition, the membrane phospholipids and their acyl moieties are important in the controlling the physicochemical properties of the biological membranes. The balanced maintenance of the arachidonic cascade pathway is well known to be critical in keeping normal mammalian cell function. On the other hand, the excess formation of some kind of eicosanoids leads to the diseases like arteriosclerosis, thrombosis, and inflammation. Dietary manipulations as well as the developments of specific inhibitors for lipooxygenases and related enzymes were effective in the prevention of such diseases. In addition to n-6 essential fatty acids, different types of dietary unsaturated fatty acids such as n-3 essential fatty acids derived from natural and processed food are included in the dietary sources. The role of n-3 essential fatty acids in peripheral tissues and cells largely remain unclear, while the function in nerve tissues was generally proposed. Hence, the specific metabolism, incorporation, and distribution of a variety of dietary unsaturated fatty acids in individual phospholipid classes must be studied in relation to the metabolism of 20:4(n-6) including the membrane level and release upon stimulation. There are several points to be considered for the cellular interaction of various unsaturated fatty acids associated with the arachidonate cascades. The regulation is not merely accounted for by the availability of 20:4(n-6), which is released by

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Abbreviations: PMA, phorbol 12-myristate 13-acetate; MDCK, Madin Darby canine kidney; PG, prostaglandin; DMEM, Dulbecco's modified Eagle medium; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CPR-I, controlled process serum replacement; DAPA, diaminopropylamine; TLC, thin-layer chromatography; 14:0, myristic acid; 14:1(n-5), myristoleic acid; 16:0, palmitic acid; 16:1(n-7), palmitoleic acid; 18:0, stearic acid; 18:1(n-9), oleic acid; 18:2(n-6), linoleic acid; 18:3(n-3), γ-linolenic acid; 18:4(n-6), arachidonic acid; 20:3(n-3), 5,8,11,14-eicosatetraenoic acid; 20:4(n-6), 11-ecosenoic acid; 20:5(n-3), 8,11-eicosadienoic acid; 20:6(n-3), dihomö-γ-linolenic acid; 20:4(n-6), arachidonic acid; 20:5(n-3), 5,8,11,14-eicosatetraenoic acid; 22:1(n-9), 13-docosenoic acid; 22:4(n-6), 7,10,13,16-docosatetraenoic acid; 22:5(n-3), 7,10,13,16,19-docosapentaenoic acid; 22:6(n-3), 4,7,10,13,16,19-docosahexaenoic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL, phosphatidylinositol; PS, phosphatidylserine; ELISA, enzyme-linked immunoassay; PBI (n–), phosphate-buffered saline without Mg²⁺ and Ca²⁺; EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.
membrane phospholipids by phospholipases.\(^4,5\) As compared to the animal experimental systems, the cell culture system is beneficial for directly studying the step influenced by dietary unsaturated fatty acids. In addition, the characteristics of cell types can be assessed by using a unique cell line having a differentiating nature.

To address the ability of mammalian cells to regulate the arachidonate cascades in the modifications with dietary unsaturated fatty acids, cultured MDCK cells were used as a model system. The MDCK cell line is derived from canine kidney tubular epithelium.\(^6\) The cultured MDCK cells are well known to activate arachidonate cascades to form cyclooxygenase products including PGF\(_2\alpha\) and PGF\(_{2\alpha}\), as major prostanoids upon stimulation with a variety of agonists such as tumor-promoting phorbol diesters,\(^7,8\) benzpyrene,\(^9\) epidermal growth factor,\(^10\) and retinoids.\(^11,12\) We have studied the action of tumor-promoting phorbol diesters in the presence of calcium ionophore A23187 for the regulation of arachidonate cascades, and provided some interesting findings. For instance, the MDCK cells responded to the simultaneous additions of 50 nM PMA and 100 nM A23187 to stimulate the production of prostanoids, requiring 24 h to reach to the maximum.\(^12\) More recently, we investigated the activation mechanism of phospholipase D that was activated through stimulation with PMA.\(^13,14\) The results indicated the lipid mediators such as phosphatidic acid and 1,2-diacylglycerol derived from the hydrolysis of phosphatidylyceroline by phospholipase D were involved in the formation of prostanoids as activators or the source of arachidonic acid.

In view of the usefulness of cultured MDCK cells, this study investigated the extent to which modifications in the content of essential fatty acids in membrane phospholipids from cultured MDCK cells can affect the activation of arachidonate cascades, and the role of each phospholipid class in the metabolism of dietary unsaturated fatty acids. The entry and release of 20:4(n-6) in membrane phospholipids were also considered in the correlation between the cellular level of 20:4(n-6) and the formation of prostanoids upon stimulation. This study has focused on the ability of cultured MDCK cells to increase or decrease a specific step in the arachidonate cascades in response to the supplementation with dietary unsaturated fatty acids as well as the enrichment of the specific phospholipid substrate pools with eicosapolyenoic acids including 20:3(n-6), 20:4(n-6), and 20:5(n-3) as sources for the synthesis of prostanoids.

Materials and Methods

**Materials.** Dulbecco's modified Eagle medium (DMEM; high glucose) with 25 mM 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid (HEPES), PMA, calcium ionophore A23187, goat anti-mouse IgG-biotin conjugate, ExtrAvidin, peroxidase diaminobenzidine (DAB)-biotin conjugate, controlled process serum replacement (CPSR-1), essentially fatty acid-free bovine serum albumin, goat anti-mouse IgG-biotin conjugate, ExtrAvidin-peroxidase conjugate, and authentic prostaglandins were purchased from Sigma (St. Louis, U.S.A.). Free saturated and unsaturated fatty acids were obtained from Sigma, Iwai Chemicals (Tokyo, Japan), and Funakoshi (Tokyo, Japan). Fetal bovine serum and newborn bovine serum were supplied by Biowhitaker (Walkersville, U.S.A.). Standard phospholipids including phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylserine (PS) were obtained from Serdary Research Laboratories (London, Ontario, Canada) and Sigma. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Funakoshi (Tokyo, Japan). Thrombin (Hi-Media, fungus powder for gas chromatography) and 3,3-dimethoxybenzidine were from Wako (Osaka, Japan), and o-phenylenediamine was from Nakarai (Kyoto, Japan). [5,6,8,9,11,12,14,15,16-H\(_2\)] Arachidonic acid (8:2TBq mmol) was obtained from New England Nuclear, Du Pont (Wilmington, U.S.A.). Sheep seminal vesicles were from Pel-Frez Biologicals (Rogers, U.S.A.). Monoclonal antibodies for PGE\(_2\),\(^16,17\) and against PG endoperoxide synthase from bovine seminal vesicles (PGE\(_2\)S\(_1\)),\(^18\) were kindly supplied by D. Y. Yamada of the Tokushima University School of Medicine (Tokushima, Japan). ImmobiIon-P transfer membrane (polyvinylidene difluoride (PVDF) membrane) was purchased from Millipore (Bedford, U.S.A.). Silica gel 60 thin-layer chromatography (TLC) plates were obtained from Merck (Darmstadt, Germany). 96-well microplates (type E) for enzyme-linked immunosorbent assay (ELISA) from Bakelite (Tokyo, Japan). Bakelite plastic 100-mm Petri dishes and 24-well plates of Corning brand for tissue cultures from Iwaki Glass (Tokyo, Japan). All other chemicals used were of reagent grade.

**Cell culture and modification with dietary unsaturated fatty acids.** The MDCK cell line, NBL-2 (JCRB829, ATCC No. CCL 34), was a 32-week-old kidney from the Cancer Research Resources Bank (JCRB) (Tokyo, Japan). For subcultures, the cells were plated at 2×10\(^4\) cells/100 mm plastic Petri dish containing 10 mL of DME HEPES medium with 10% fetal bovine serum (Medium A). The cells were grown for 3-4 days to confluence at 37 °C in a humidified atmosphere of 7% CO\(_2\) in air. For the subculture experiment, the culture medium was replaced with the delipidated medium with 5% CPSR-1 to which dietary unsaturated fatty acid-free BSA to give a final concentration of 100 μM. The cells were grown to confluence for additional 2 days. Stock solutions of dietary unsaturated fatty acids at 100 μM were prepared by dissolving in ethanol and stored at −20 °C. For the preparation of 1 μM complex of individual unsaturated fatty acid and essentially fatty acid-free BSA, one volume of a stock solution of 100 μM unsaturated fatty acids was mixed with 100 volumes of 1 μM BSA in the DME HEPES medium.

For the modification with cicosanoid synthesis with 50 μM PMA and 100 nM A23187 after modification with dietary unsaturated fatty acids, the culture medium was replaced by the DME HEPES medium with 5% CPSR-1 to which dietary unsaturated fatty acid-free BSA was added to give a final concentration of 10 μM. The cells were incubated in a 2% CO\(_2\) incubator as described above. PMA and A23187 were prepared as stock solutions of 50 μM and 100 μM in dimethyl sulfoxide, respectively.

**Analysis of the composition of phospholipids and fatty acids.** After modification with dietary unsaturated fatty acids, the cultured monolayer cells were washed with phosphate-buffered saline without Ca\(^2+\) and Mg\(^2+\) (PBS−)\(^−\). Then, the monolayer cells were scraped off the 100-mm plastic dishes with a Teflon-made policeman into 2 mL of ice-cold methanol. The scraped cell suspension was transferred to a glass tube to which 1 mL of chloroform and 0.8 mL of an aqueous solution of 0.2 M KC\(_2\)HPO\(_4\) and 5 mM EDTA were added. The monophasic mixture was mixed vigorously by a vortex mixer and centrifuged at 1200 × g for 5 min at room temperature. The supernatant was taken to another tube and the phase separation was done essentially by the method of Bligh and Dyer.\(^19\) The lower chloroform phase was evaporated to dryness under a vacuum using a Taife centrifugal concentrator, model VC-50. The dried materials were dissolved in 100 μL of chloroform methanol (2:1, v:v), and a 25 μL portion was spotted on silica gel 60 plates for thin-layer chromatography (TLC). The separation of individual phospholipids was done by two-dimensional TLC using chloroform methanol 25% ammonium hydroxide water (65:35:5:0.6, v:v) in the first dimension and chloroform methanol acetic acid water (45:19:10:5.4, v:v) in the second dimension. Spots were measured densitometrically at 550 nm by a Shimadzu TLC scanner, model CS-920 after they were sprayed with a solution of 10% cupric sulfate and 8% phosphoric acid and heated at 180 °C for 20 min.

For the analysis of the fatty acid composition of phospholipid classes, the individual phospholipids were isolated by two-dimensional TLC on silica gel 60 plates as described above. The phospholipid bands corresponding to the desired phospholipids were made visible by spraying
with 0.01% primuline in 80% acetone and exposed to a long-wavelength UV light (365 nm). The scraped band was used for the preparation of fatty acid methyl esters from each phospholipid class. The spots scraped off the TLC plates were directly allowed to react with 0.5 ml of 2.5% H$_2$SO$_4$ in a mixture of methanol-benzene (9:1, v/v) at 80°C for 1 h in a screw-capped glass tube. The fatty acid methyl esters were extracted with hexane, evaporated to dryness, and redissolved in hexane including 0.01% butylated hydroxytoluene as an antioxidant. A 2-μl portion was analyzed by gas chromatography on a Hitachi gas chromatograph apparatus, model 263-30 with a 3-mm internal diameter x 2-m stainless column packed with 10% (w/w) Thermosil-1000 on Chromosorb W (80-100 mesh), acid-washed and silanized (AW-DMCS) and flame ionization detector. Nitrogen gas was used as a carrier gas at a flow rate of 66 ml/min. The temperature of the column oven, injection port, and detector were set at 245 C, 260 C, and 260 C, respectively. Peak areas were calculated using a Shimadzu integrator, model C-ElB.

**Enzyme-linked immunosorbent assay (ELISA) of PGE$_2$.** The level of PGE$_2$ was measured by a competitive assay between the immobilized antigen and a standard compound or the sample from the culture medium as described recently. The conjugates of PGE$_2$ and proteins like BSA for generating antibodies in mice and bovine γ-globulin for the immobilized antigen in ELISA were prepared by the method using N-succinimidyl ester. Monoclonal antibodies against PGE$_2$ were prepared by using a hybridoma technique between a myeloma cell line, Sp2/0-Ag14 and lymphocytes from BALB/c mice immunized with the conjugate of PGE$_2$ and bovine serum albumin as described before. A monoclonal antibody used here showed cross-reactions of 3.3% and 60.6% for PGE$_1$ and PGE$_3$, respectively. There were cross-reactions of less than 0.1% for other classical prostanoids.

For the assay, the conjugate of PGE$_2$ and bovine γ-globulin was used as an immobilized antigen in a 96-well dish, which was allowed to competitively react with monoclonal antibody against PGE$_2$ in the presence of 2-fold serial dilutions containing authentic PGE$_2$ from 5 pg to 10 ng or the sample. In addition, goat anti-mouse IgG-biotin conjugate was added to give a further immunocomplex and followed by the reaction with ExtrAvidin and then peroxidase DAPA-biotin conjugate sequential reagents. The enzyme reaction was done at 30°C for 20 min by incubating with 100 μl of 18.5 mM o-phenylenediamine and 3.53 mM H$_2$O$_2$ as substrates in 50 mM citrate-phosphate buffer at pH 5. One half volume of 5% H$_2$SO$_4$ was added to the reaction mixture and the absorbance at 492 nm for 405 nm as a reference was measured by a Foss ELISA microplate reader, model MPAR4. Other details of the procedures were described in our previous report.

**Assay of PGE$_2$ synthetase activity from arachidonic acid.** After treatment with stimulators, the confluent monolayer cells were washed with cold PBS (pH 7.4) and resuspended in 100 μl of 0.2 M Tris- HCl buffer at pH 8 containing 2 μM hematin, 5 mM tryptophan, 2 mM glutathione, and 20 μM [1-14C]arachidonic acid (20,000 cpm/mmol) as a substrate. The reaction was done at 25°C for 30 min. The reaction products were extracted and separated by TLC on silica gel plates. The spots corresponding to PGE$_2$ were scraped off, and then the radioactivity was measured by liquid scintillation counting. Details were as reported earlier.

**Western blot analysis of PG endoperoxide synthase.** The monolayer cells were stimulated in the presence or absence of both 50 nM PMA and 100 nM A23187 for 3 h and 24 h. To obtain the solubilized whole cell preparations, the treated cells were scraped into PBS (−) containing 1 mM EDTA. The cells were collected by centrifugation at 1200 x g for 5 min at 4°C. The pellets were dispersed in the ice-cold homogenization buffer containing 50 mM Tris- HCl buffer at pH 8, 10 mM EDTA, 10 mM phenylmethysulfonyl fluoride, and 1% Tween 20. The cell suspension was sonicated at 20 kHz for 15 min six times. After centrifugation at 105,000 x g for 20 min at 4°C, the supernatant was used a solubilized cell preparation including an enzyme source.

The solubilized cell preparations of cultured MDCK cells (70 μg protein) and sheep seminal vesicles (20 μg) as a control were electrophoresed on a sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) on a 7.5% acrylamide gel and a 2.5% stacking gel in the presence of 0.1% SDS. The resolved proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane in 100 mM Tris and 192 mM glycine. Thereafter, the PVDF membrane was immersed for 1 h in PBS (−) containing 1% BSA and 0.02% NaN$_3$ for blocking the nonspecific binding of other proteins. The blots were washed with PBS (−) containing 0.05% Tween 20 and 0.02% NaN$_3$, and then incubated for 1 h at room temperature followed by overnight at 4°C with a 100-fold diluted solution of monoclonal antibody (PES-7) against PG endoperoxide synthase from bovine seminal vesicles. After washing as above, the blots were incubated with a 100-fold diluted goat anti-mouse IgG-biotin conjugate in PBS (−) containing 0.5% BSA and 0.02% NaN$_3$ at 25°C for 2 h. After washing, the membrane was incubated with a 1000-fold diluted solution of ExtrAvidin-peroxidase conjugate in PBS (−) containing 1% BSA followed by incubation at 25°C for 30 min. The blots were washed and developed with a solution for peroxidase substrate containing 50 mM Tris-HCl buffer at pH 7.6, 0.01% H$_2$O$_2$, and 0.83 mM 3,3'-diaminobenzidine at 25°C for 1 h. The membrane was washed with distilled water to stop the reaction.

**Evaluation of cell proliferation and cytotoxicity.** The MTT assay was done as reported before. The cultured MDCK cells were modified with 100 μM dietary unsaturated fatty acids for 3 days. To a 24-well dish containing 100 μl of culture medium, 10 μl of a 5 mg/ml solution of MTT in PBS (−) was added and mixed vigorously. After incubation at 37°C for 4 h, 100 μl of 0.04% HCl in isopropanol was added and mixed again. The mixture was left at room temperature to ensure that the dark blue crystals were dissolved thoroughly. The absorbance at 564 nm was measured within 1 h of the addition of isopropanol.

**Other methods.** Protein was measured with BSA as a standard by the method of Lowry et al. or Markwell et al. which is suitable for measuring membrane proteins.

**Data treatment.** Assays were done in triplicate unless otherwise indicated. Data values were expressed as means ± S.E. (standard error) from a representative experiment done at least three times.

**Results**

**Modification of cultured MDCK cells with dietary unsaturated fatty acids.** The confluent monolayer MDCK cells were cultured for 2 days in the delipidated medium with various types of dietary unsaturated fatty acids at 100 μM. The total phospholipids were extracted, separated to individual phospholipids by TLC, and measured by a densitometric assay. The analysis of phospholipid composition showed that the orders from the more abundant phospholipid classes in all of modified cells were PC (48.56%), PE (26.36%), PI (3.9%), and PS (2.7%) as many types of other mammalian cells.

The supplementation of individual unsaturated fatty acids was done as an equal molar complex of the unsaturated fatty acid and essentially fatty-acid free BSA. The colorimetric MTT assay was done to evaluate if the modification of dietary unsaturated fatty acids might influence the cell proliferation and cytotoxicity of the cultured MDCK cells. The values of the MTT assay were linearly correlated with cell numbers of cultured MDCK cells, no significant cytotoxicity was recognized as shown in Fig. 1. Instead, n-3 essential fatty acids like ω-3linoleic acid (18:3(n-3)) and 20:5(n-3) except 4,7,10,13,16,19-docosahexaenoic acid (22:6(n-3)) stimulated cell proliferation in modified MDCK cells. There was no stimulation with other n-6 and n-3 unsaturated fatty acids.

**Specificity of elongation, desaturation, and incorporation of dietary unsaturated fatty acids into membrane phospholipids in modified cells.** To analyze the distribution of dietary unsaturated fatty acids in individual phospholipid species, data were calculated by considering the phospholipid composition of the cultured MDCK cells modified with either n-6 and n-3...
unsaturated fatty acids as shown in Fig. 2. C18-Fatty acids such as 18:2(n-6), 18:3(n-6), and 18:3(n-3) were more selectively incorporated into PC than any other phospholipids. Compared with 18:2(n-6), 18:3(n-6) was more effectively converted to the elongated and desaturated products like dihomo-ω-6-linolenic acid (20:3(n-6)) and 20:4(n-6). PC has the highest content of 20:3(n-6) derived from 18:3(n-6) than other phospholipid species. In contrast, 20:4(n-6) that was generated from both 18:2(n-6) and 18:3(n-6) was the most abundant in PE. Supplementation of 20:4(n-6) increased the level of 22:4(n-6) predominantly in PE and PS.

As for the n-3 unsaturated fatty acids, 18:3(n-3) was largely incorporated into PC. 20:5(n-3) was produced as the metabolite of 18:3(n-3), but the further metabolized 7,10,13,16,19-docosapentaenoic acid (22:5(n-3)) was hardly detected from 18:3(n-3). However, the supplementation of 20:5(n-3) was more effective to convert it to 22:5(n-5). Almost no further elongated 22:6(n-6) was recognized, reflecting the very low Δ4 desaturase activity in cultured MDCK cells. When the cells were modified with 22:6(n-3), the highest proportion of 22:6(n-3) was obtained in PE at 14.9% with 9–10% levels in PC and PI. 20:5(n-3) was formed from 22:6(n-3) by a retroconversion involving peroxisomal enzymes, as reported previously.251 PC, PE, and PI had levels of 6–10% of the retroconverted 20:5(n-3), but there was no 20:5(n-3) in PS.

**Arachidonic acid level in membrane phospholipids in modified cells**

Changes in the level of 20:4(n-6) in membrane phospholipids from cultured MDCK cells modified with various dietary unsaturated fatty acids are presented in Fig. 3. Based on the data on the compositions of phospholipids and fatty acids, the changes in the 20:4(n-6) level from unmodified cells to the corresponding modified cells were calculated, reflecting the distribution of 20:4(n-6) in each phospholipid class from the modified cells among total cellular 20:4(n-6). In PC and PE, the supplementation of 20:4(n-6) was the most effective to increase the level of 20:4(n-6) level to almost the same extent. 18:3(n-6) was comparable to 20:4(n-6) to raise the level of 20:4(n-6) in PE, but not in PC which had half of 20:4(n-6) in PE. PC had more than two times less 20:4(n-6) level in 18:2(n-6) or

![Graph showing the incorporation of fatty acids into membrane phospholipids](image-url)
18:3(n-6)-modified cells than in 20:4(n-6)-modified cells. There were almost no change in the 20:4(n-6) level in PI and PS following the modification. The modification with n-3 essential fatty acids decreased the level of 20:4(n-6) in PE.

Stimulation of PGE₂ synthesis with PMA and A23187 in cultured MDCK cells following modification with dietary unsaturated fatty acids

A mixture of 30 nM PMA and 100 nM A23187 were found to stimulate the PGE₂ synthesis synergistically in cultured MDCK cells as described previously.1,2 Our previous report also showed that the incubation for almost 24 h was necessary for the PGE₂ synthesis to reach the maximal level. This study examined the availability of 20:4(n-6) for the synthesis of prostanoids following modification of cultured MDCK cells with various unsaturated fatty acids (Fig. 4). n-6 Essential fatty acids like 18:2(n-6), 18:3(n-6), and 20:4(n-6) significantly stimulated the synthesis of PGE₂. The effects of 18:2(n-6) and 18:3(n-6) were slightly lower, but almost comparable to that of 20:4(n-6) which was the most effective. There was a slight increase in the formation of immunoreactive PGE₂ with the supplementation of n-3 essential fatty acids such as 18:3(n-3) and 20:5(n-3). This might reflect the production of the 3 series of PGE from 20:5(n-3).

Regulation of PGE₂ synthetic activity from arachidonic acid in modified cells

We have previously shown using cultured MDCK cells that the greatest stimulation of PGE₂ synthesis occurs in the simultaneous presence of both PMA and A23187 at concentrations that are less active by either compound alone. It took more than 24 h for PGE₂ formation to reach the maximum. This was found to be caused by the slower induction of PG endoperoxide synthase through the action of PMA as reported before.13,14 This study also examined the time course of PGE₂ synthetic activity from arachidonic acid in the presence or absence of both 50 nM PMA and 100 nM A23187, reflecting the activation of PG endoperoxide synthase activity. The activation of PG endoper-
oxide synthase activity was clearly recognized at around 3h and reached to the maximum at 24h. The increase in the immunoreactive band at about 70 kDa corresponding to de novo synthesis of PG endoperoxide synthase was detected by Western blot analysis by using specific antibodies for the enzyme from bovine seminal vesicles.

The cultured MDCK cells were modified for 2 days with dietary unsaturated fatty acids and then stimulated with or without 50 nM PMA and 100 nM A23187 for additional 2 days (Fig. 5). Compared with the unstimulated cells, there were significant increases in the PGE2 synthetic activities from arachidonic acids upon stimulation with agonists in the cells modified with dietary unsaturated fatty acids such as C18 unsaturated fatty acids and 22:6(n-3). However, n-6 essential fatty acids including 20:3(n-6) and 20:4(n-6) abolished the agonist-stimulated PGE2 synthesis to the levels of unstimulated cells. The supplementation with 20:5(n-3) was also inhibitory.

Discussion

The approximately half confluent MDCK cells were modified in delipidated medium supplemented with dietary unsaturated fatty acids including essential fatty acids. There were some differences in the composition of phospholipids among the modified cells. However, no rule was detected among them. Some enzyme step leading to the synthesis of phospholipids might be influenced by the modification with dietary unsaturated fatty acids. To minimize the cytotoxicity, the supplementation of dietary unsaturated fatty acids to the culture medium was done in an equal molar complex with essential fatty acid-free BSA. Furthermore, to evaluate the cytotoxicity and the effects of proliferating activity of dietary unsaturated fatty acids, the colorimetric MTT assay was done as described in Fig. 1. The stimulation of cell growth in the modified cultured MDCK cells was found with 18:3(n-3) and 20:5(n-3). In contrast, there was neither stimulation nor inhibition with 22:6(n-3) even though they are the same n-3 essential fatty acids. In this study, no mechanistic details were known. The metabolic and functional responses in the cultured MDCK cells relating to renal function remained to be identified. Long chain n-3 polyunsaturated fatty acids have been reported to play a role in the prevention of a number of diseases such as cancer, heart disease, and arthritis. A recent report using a cell culture system has shown that 22:6(n-3) as a free fatty acid or a component of mixed-chained PC was effective in making tumor plasma membranes significantly more permeable, which may partly explain its anti-tumor properties. Since little is known about the role of n-3 unsaturated fatty acids in renal cells or tissues including cultured MDCK cells, further studies need to be done at the cellular and molecular levels.

The cultured MDCK cells in this study were modified for 2 days in the culture medium supplemented with various dietary unsaturated fatty acids. The length of the incubation for the modification with unsaturated fatty acids might be long enough to equilibrate the distribution of the supplemented dietary unsaturated fatty acids. The distribution of polyunsaturated fatty acids has been proposed to be decided by the remodeling processes mediated by CoA-independent transacyclases and CoA-dependent transacyclases. However, these enzymes were mostly responsible for the transacylation of arachidonic acid. No other transacyclases have been reported for other polyunsaturated fatty acids. Our research examined the changes in the fatty acid composition of each phospholipid class, and focused on the specificity of the metabolism of the unsaturated fatty acids that were incorporated into individual phospholipids (Fig. 2). The analysis of specific metabolism, entry, and remodeling of unsaturated fatty acids in each phospholipid class might provide useful information on the role of the specific phospholipids in the membrane function mediating eicosanoid productions or other lipid biofactors derived from biological membranes.

Our results indicated that C18 unsaturated fatty acids such as 18:2(n-6), 18:3(n-6), and 18:3(n-3) were more selectively incorporated into PC than PE, but 20:4(n-6) and 22 unsaturated fatty acids showed the preference for PE rather than PC (Fig. 2). Inconsistent with 20:4(n-6), the supplemented 20:5(n-5) was instead more efficiently esterified in PC than in PE. However, the higher level of 20:5(n-5) derived from 18:3(n-3) was present in PE rather than PC. The mechanism of this differentiation is unclear at present. The selective transacylase system specific for 20:5(n-3) might possibly be operative for the transfer of the esterified 20:5(n-3). In addition, the supplementation of 18:3(n-6) resulted in a more efficient conversion into 20:4(n-6) than that of 18:2(n-6). The resulting 20:3(n-6) and 20:4(n-6) were preferentially esterified in PC and PE, respectively. The selectivity of 20:3(n-6) for PC was also seen in 18:2(n-6)-modified cells. The difference of 20:3(n-6) and 20:4(n-6) levels between PC and PE might affect the availability of the substrates for eicosanoid synthesis upon stimulation in cultured MDCK cells. The supplementation of 18:3(n-6) and 20:4(n-6) generated the 22:4(n-6) as the elongated products. However, 18:2(n-6) was much less efficient for that. A possible explanation is that 18:2(n-6) is more susceptible to β-oxidation. Therefore, 18:2(n-6) was not as efficiently retained as other homologues like 18:3(n-6) and 20:4(n-6). However, as presented in Fig. 2, the incorporation of 18:2(n-6) and 18:3(n-6) was at the same level, suggesting that the preferential incorporation of 18:2(n-6) into PC was more efficient than its further metabolism to 18:3(n-6) and 20:4(n-6). Once the 18:2(n-6) was esterified in PC, the additional conversion might not be as efficient. The metabolism of the supplemented 18:3(n-3) or 20:5(n-3) to 22:5(n-3) was minor. Almost no 22:6(n-3) was detected. In these MDCK cells, the reactions of elongation from 20:5(n-3) and Δ4 desaturation were less active. Whether these reflect the characteristics of the kidney epithelial cells or MDCK cell lines is not known. A previous study has shown that 22:5(n-3) was metabolized in rat liver via a pathway that is independent of a Δ4 desaturase activity. This response might be associated with membrane fluidity. Previously, the retroconversion of 22:6(n-3) to 20:5(n-3) was found to be done by peroxisomal function in isolated rat liver cells. The importance of this pathway was also pointed out for 22:4(n-6), from which 20:4(n-6) can be replenished in endothelial cells and fibroblasts.

Arachidonic acid levels in membrane phospholipids
were calculated from the data of compositions of phospholipids and fatty acids (Fig. 3). The 20:4(n-6) levels reflect the changes between the modified and unmodified cells. As expected, the supplementation of 20:4(n-6) was most effective to raise the 20:4(n-6) level in every phospholipid class. In PE, 18:3(n-6) increased the 20:4(n-6) level by as much as the supplementation of 20:4(n-6). In sharp contrast, the modification with 18:2(n-6) was more than 2-fold lower to increase the 20:4(n-6) in PC from the membranes. The more abundant 20:4(n-6) in PE than in PC may be accounted for by the other findings that arachidonoyl transacylases transfer 20:4(n-6) from PC to lysophosphatidylethanolamine, including lysoplasmenyl-ethanolamine in human platelets, or in rabbit alveolar macrophages. More studies have yet to be done about the transacylases specific for 20:4(n-6) in these MDCK cells. On the other hand, PI and PS were much ineffective in accumulating 20:4(n-6). Thus, this led us to suggest that PI might not be involved in the source of the eicosanoid synthesis upon stimulation in these cultured MDCK cells. These findings seemed to be different from the previous studies using endothelial cells which have indicated that the cells maintain a critical level of 20:4(n-6) in PI. Also, evidence suggesting that PE was a major donor of polyunsaturated fatty acids for PGE synthesis was reported in a bradykinin-stimulated mouse fibroblastic cell line, EFD-1 cells. Nevertheless, the relative level of 20:4(n-6) in our 20:4(n-6)-modified MDCK cells still high although 20:4(n-6) in PI did not constitute the main components of total 20:4(n-6). The specific pools of 20:4(n-6) cannot be completely excluded in view of the fact that the 20:4(n-6) available for the synthesis of eicosanoids was much less than the total 20:4(n-6) in membrane phospholipids.

Our studies have previously reported that prostaglandin synthesis in cultured MDCK cells was stimulated synergistically in the presence of both 50 nM PMA and 100 nM A23187. We have also shown that the stimulated PGE synthesis was mediated by the induction of PG endoperoxide synthase activity caused by the addition of PMA. When the MDCK cells were modified with various dietary unsaturated fatty acids, n-6 essential fatty acids such as 18:2(n-6), 18:3(n-6), and 20:4(n-6) formed PGE efficiently upon stimulation with PMA and A23187 (Fig. 4). The potent effect was the supplementation with 20:4(n-6) as expected. The finding noted here was that the effects of 18:2(n-6) and 18:3(n-6) were almost comparable to 20:4(n-6) even though less efficient in the 20:4(n-6) level in membrane phospholipids. Considering the result of the 20:4(n-6) levels in Fig. 3, the source for the substrate of prostanooids was strongly suggested to be predominantly provided by PE rather than PC. In accordance with our study, the cultured MDCK cells have been earlier reported to release 20:4(n-6) upon the early stimulation of prostanooid formation within 15 min by PMA from cellular phospholipids including PE, PC, PI, and PS. However, the major donor of 20:4(n-6) has been shown to be the PE fraction from which both the diacyl-PE and 1-alk-1'-enyl-2-glycerol-3-phosphoethanolamine are 20:4(n-6) donors. In this study, there was some difference in the 20:4(n-6) level between 18:2(n-6) and 18:3(n-6) in PE (Fig. 3). However, the formed PGE levels were almost the same (Fig. 4). A possible reason for this discrepancy is that the 20:4(n-6) pool for the PGE synthesis was nearly saturated in PE. Monoclonal antibody for PGE2 has cross-reactions with PGF2 and PGE3. Slight increases in immunoreactive PGE2 in Fig. 4 might reflect the synthesis of PGE2 formed in the MDCK cells modified with n-3 essential fatty acids upon the stimulation.

Our attention was especially paid to the regulation of the arachidonate cascade reaction in the cultured MDCK cells modified with various dietary unsaturated fatty acids as well as the enrichment of each phospholipid class with dietary unsaturated fatty acids. As shown in Fig. 5, this study used the cultured MDCK cells showed that the cells modified with eicosapolyenoic acids like 20:3(n-6), 20:4(n-6), and 20:5(n-6) failed to activate the PGE2 synthetic activity from 20:4(n-6), reflecting PG endoperoxide synthase activity. As shown in Fig. 1, this inhibition was not accounted for by the cytotoxicity of these eicosapolyenoic acids. Both n-6 and n-3 unsaturated fatty acids were involved, so that the inhibitory mechanism could not also be explained by the specific metabolites like one of eicosanoids. A previous study has reported that the supplementation of 18:2(n-6) to the endothelial cells inhibited the capacity of the cells to produce PGI2 by decreasing 20:4(n-6) level of cellular phospholipids. This might reflect the lower capacity of the endothelial cells to convert 18:2(n-6) to 20:4(n-6) or susceptibility to the inhibition by 18:2(n-6) or other polyunsaturated fatty acids. As opposed to the endothelial cells, the modification with 18:2(n-6) had almost the same effect as 20:4(n-6) in stimulating PGE2 synthesis in our experimental system. Similarly, when Swiss mouse 3T3 cells were stimulated for 5 min with A23187, the PGE2 production were found to increase as a result of 18:2(n-6) supplementation. In addition, the supplementation of cultured MDCK cells with 18:2(n-6) increased bradykinin-stimulated PGE2 synthesis within 5 min by several-fold. On the other hand, the supplementation with 20:4(n-6) was reported not to increase the bradykinin-stimulated PGE2 formation even though the 20:4(n-6) content increased by about 9-fold. Currently, we showed the significant increase in the production of PGE2 during long-term incubations taking more than 24 h. Hence, the earlier works appeared not to see the maximal activation of arachidonate cascades because of short-term experiments although the agonists used were not the same as ours.

Deng et al. have earlier reported an increased ability of Swiss mouse 3T3 cells to synthesize PGE2 from exogenous 20:4(n-6) when the cells were enriched with 18:2(n-6), suggesting the activation of prostanooid-forming enzymes. However, we were not able to show evidence that the modification with some species of dietary unsaturated fatty acids increased the induction of the PG endoperoxide synthase activity as compared with unmodified cells. The modification with 20:3(n-6), 20:4(n-6), and 20:5(n-3) abolished the activation of the enzyme activity (Fig. 5). This might reflect negative feed-back regulation of the cultured MDCK cells to repress the prostaglandin synthesis upon stimulation. In accordance with our findings, Lewis et al. have earlier reported that the prolonged exposure of cultured MDCK cells to 20:4(n-6) during modification inhibits the bradykinin-stimulated prostanooid synthesis within 5 min. Since this study only examined the
prostanoid synthesis upon stimulation during short-term incubations, this result is not necessarily comparable with our findings. However, they observed the suppressed use of exogenous 20:4(n-6) in the cultures that were supplemented with 20:4(n-6) during growth, suggesting the decreased ability of forming PGE$_2$ synthetic activity. Thus, the modification of cultured MDCK cells with 20:4(n-6) seemed to be similiar inhibitory for subsequent stimulations with agonists by both short-term and long-term incubations. One possible effect might be autocline control of the produced PGE$_2$, or inactivation of PG endoperoxide synthases by fatty acid hydroperoxides derived from 20:4(n-6). Recent molecular cloning techniques showed that PG endoperoxide synthase exists in two isofoms, PG endoperoxide synthase-1 and -2. The former was constitutive while the latter was transiently inducible by several agonists.22,43 Therefore, it is reasonable to assume the involvement of the type-2 enzyme in our MDCK cells. However, additional molecular and cellular studies were required to establish the above mechanism by considering some variations depending on the mammalian cell types. Furthermore, the functional alterations have yet to be clarified in cultured MDCK cells and other mammalian cells induced by the modifications with dietary unsaturated fatty acids. This kind of research is expected to elucidate the novel cellular response caused by dietary regulation mediating essential fatty acids and eicosanoids.

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