Fatty Acid Metabolism and Cell Proliferation. III. Effect of Prostaglandin Biosynthesis either from Exogenous Fatty Acid or Endogenous Fatty Acid Release with Hydralazine

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ABSTRACT

Primary cultures of smooth muscle cells were established from the medial layer of guinea pig aorta. Cells were seeded at from 40 to 80 cells per cm^2 and cloned for 8 days. Media were analyzed for PGI_2 (6-keto-$PGF_{1\alpha}$) using radioimmunoassay. Prostanoids were synthesized when cells were grown in media alone. Arachidonic acid stimulated prostanoid synthesis and promoted cell proliferation. Indomethacin blocked prostanoid synthesis and abolished the stimulatory effect of arachidonic acid on cell proliferation. Hydralazine stimulated fatty acid release and prostanoid synthesis in confluent cells. Hydralazine also stimulated prostanoid synthesis and promoted proliferation in growing cells. Indomethacin blocked prostanoid synthesis and abolished the stimulatory effect of hydralazine on cell proliferation.
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INTRODUCTION

Several studies have shown that low concentrations of arachidonic acid [20:4(n-6)] promote the proliferation of a number of cell lines in tissue culture. These cell lines include HeLa cells (1), XS 63.5 cells (2), 7,12-dimethylbenz(a)anthracene tumor cells (3,4) and smooth muscle cells (5). Arachidonic acid is the precursor of prostaglandins and prostacyclin (PGI_2). A number of studies have shown that prostaglandins either promote (5-8) or inhibit (5-7,9-12) cell proliferation depending on the concentration of prostaglandin added to, or generated by, the cells in tissue culture. Other studies have suggested that PGI_2 either promotes (13) or inhibits (14,15) cell proliferation. In the present investigation, we have examined the effects of exogenous 20:4(n-6) and endogenous 20:4(n-6) release on the cellular biosynthesis of prostaglandins and on the proliferation of aorta smooth muscle cells.

MATERIALS AND METHODS

Materials

Arachidonic acid was purchased from NuChek (Elysian, MN) and was shown to be peroxide-free by thin layer chromatography (5). Hydralazine HCl and indomethacin were purchased from Sigma Chemical Co. (St. Louis, MO). Reference prostanoids were kindly supplied by Dr. J. Pike. Antiserum for the radioimmunoassay (RIA) of 6-keto-$PGF_{1\alpha}$ was kindly supplied by Dr. L. Levine. Antimyosin (Chicken gizzard) was kindly supplied by Dr. U.G. Stewart.

Tissue Culture

Primary cultures of smooth muscle cells were established from the dissected medial layer of guinea pig aorta from prepubertal males (5,16). Smooth muscle cells were identified by their reactivity to antibodies prepared from smooth gizzard muscle (17). The medium used for growing cells to confluency (growth medium) was prepared from 1X Eagle's minimum essential medium containing Hank's salts and 25 mM HEPES buffer (GIBCO, Grand Island, NY) supplemented with 50 μg per ml gentamycin sulfate (Schering, Kenilworth, NJ), 2 mM glutamine, 1X nonessential amino acids (Microbiological Associates, Walkersville, MD), 1 mM pyruvate, and 1.3 mg per ml of sodium bicarbonate. This medium was supplemented with 10% fetal bovine serum (FBS) (Sterile Systems, Logan, UT: Hyclone, lots 100331 and 100348). The medium in cell proliferation and prostanoid experiments (experimental medium) consisted of growth medium supplemented with either 10% or 20% FBS, 1X essential amino acids, and essential vitamins.

Arachidonic acid was dissolved in 95% ethanol and diluted 1:500 with experimental medium. Hydralazine HCl and indomethacin were dissolved in 95% ethanol and diluted 1:2500 with the medium. Control cultures were treated with experimental medium containing the same amount of ethanol.

Cell Proliferation

Smooth muscle cells, passage number 4 and 3-5 days postconfluent, were seeded at low densities (from 40 to 80 cells per cm^2) in Falcon single-well plates (60 × 15 mm) or Costar tissue culture dishes (60 × 15 mm). Cells were allowed to attach to the plastic petri plates for one day before initial treatments. Cells were retreated with a media change at day 5 of the incubation period. After an 8-day incubation period, cells were fixed in 2.5% phosphate-buffered glutaraldehyde or 3.7% phos-

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phate-buffered formalin and stained with filtered Giemsa.

Total cell area was measured by image analysis using the Optomax Visual Analysis System. A relative cell count was obtained from the total cell area on the plate. The relationship between cell area and cell number was validated both with a microscope (18) and with a Coulter counter (19). When cultures were grown in medium alone, typical Coulter counter data gave 137,000 ± 6,600 cells per plate. The cell number depended on the primary culture and the source of FBS. Cells from the same primary cultures and batch of growth medium were compared in each treatment series.

Prostaglandin and Prostacyclin Biosynthesis

Media were obtained from confluent cultures (Corning T-25 flasks) or cloning cultures (Costar tissue culture dishes). Media aliquots from eight Costar dishes were pooled for analysis in cloning experiments. PGI_2 was measured as the 6-keto-$PGF_{1\alpha}$ metabolite by a standard RIA procedure (20). The cross-reactivity of the 6-keto-$PGF_{1\alpha}$ antibody was: PGE_2, 0.15%; PGD_2, 0.02%; $PGF_{2\alpha}$, 0.10%; arachidonic acid, 0.005%. Data for 6-keto-$PGF_{1\alpha}$ are reported both as nmol/culture and relative concentration in percent.

RESULTS AND DISCUSSION

Effect of Fatty Acids on Cell Proliferation

Cells were cloned in media alone or media containing 10 μM 18:2(n-6), 20:3(n-6) or 20:4(n-6). These fatty acids were not interconverted because smooth muscle cells in culture have no desaturase activity (21,22). Only 20:4(n-6) enhanced cell proliferation (Fig. 1). Previous studies from our laboratory show that prostanoids, synthesized from 20:4(n-6) but not 20:3(n-6), enhance smooth muscle cell proliferation (5,23). The data in Figure 1 suggest but do not prove that 20:4(n-6) stimulates cell proliferation through prostanoid synthesis rather than a generalized fatty acid effect that would be found with other related polyunsaturated fatty acids.

Prostanoid Biosynthesis in Cell Cultures

Previous studies have shown that hydralazine enhances prostanoid biosynthesis in tissues and cell cultures (24,25). We find that hydralazine enhances prostanoid biosynthesis (6-keto-$PGF_{1\alpha}$) when it is added in media alone to confluent smooth muscle cells (Fig. 2). Hydralazine does not stimulate prostanoid synthesis when it is added in media together with excess 20:4(n-6) (Fig. 2). Similar results are obtained with 6-keto-$PGF_{1\alpha}$ and PGE_2 (data not shown). These data show that hydralazine

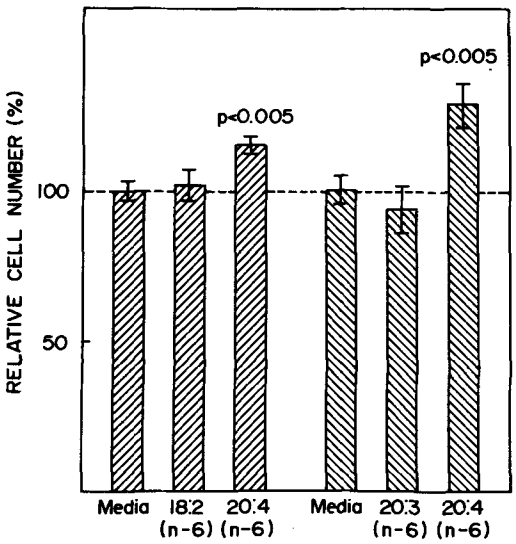

FIG. 1. Effect of 18:2(n-6) and 20:3(n-6) and 20:4(n-6) on cell proliferation. Cells were cloned for 8 days in Costar tissue culture dishes containing media alone or media supplemented with 10 μM fatty acid. Each group contained 8 plates. Data are expressed as relative cell number compared to control (media alone). Vertical lines show SEM. Treatment groups that differed significantly (Student t-test) from the control group are noted in the figure.

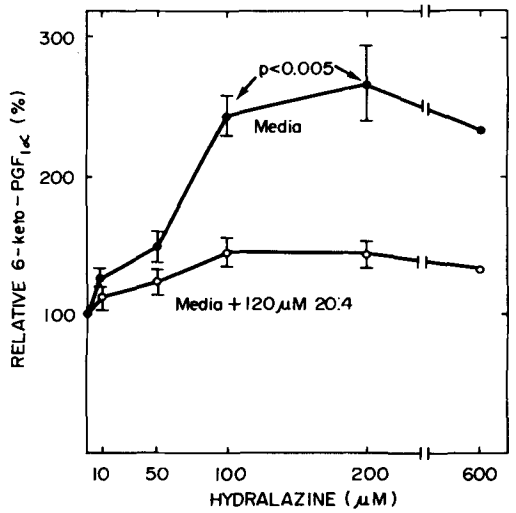

FIG. 2. Effect of hydralazine concentration on PGI_2 (6-keto-$PGF_{1\alpha}$) biosynthesis in confluent cultures of smooth muscle cells incubated for 24 hr in media alone or media containing 120 μM 20:4(n-6). Data are expressed as relative 6-keto-$PGF_{1\alpha}$ amount compared to control (media without hydralazine). Control cultures contained 285 ± 51 pmol/culture with media alone and 2190 ± 280 pmol/culture with 120 μM 20:4(n-6). Data represent mean ± SEM for 8 different primary cultures. Vertical lines show SEM. Significant differences (Student t-test) are noted in the figure.

stimulates fatty acid release rather than fatty acid conversion to prostanoids.

Exogenous 20:4(n-6) and endogenous fatty acid release through hydralazine both enhance 6-keto-$PGF_{1}\alpha$ levels in growing cultures (Fig. 3). Indomethacin blocks these effects. Similar results are obtained for 6-keto-$PGF_{1}\alpha$ and PGE_2 (data not shown).

Relationships between Prostanoid Biosynthesis and Cell Proliferation

Exogenous 20:4(n-6) (Figs. 1 and 4) and hydralazine (Fig. 5) both stimulate cell proliferation. Indomethacin blocks the stimulatory effects of 20:4(n-6) and hydralazine on cell proliferation (Figs. 4 and 5) just as it blocks enhanced prostanoid synthesis in these cultures (Fig. 3).

It is difficult to establish a causal relationship leading from an effector through prostaglandin biosynthesis to cell proliferation. Studies with exogenous 20:4(n-6) and endogenous fatty acid release through hydralazine indicate a causal relationship and other studies in the recent literature support this hypothesis. For example, vasopressin enhances prostaglandin synthesis in cell cultures (26) and this agent promotes the proliferation of 3T3 fibroblasts (27).

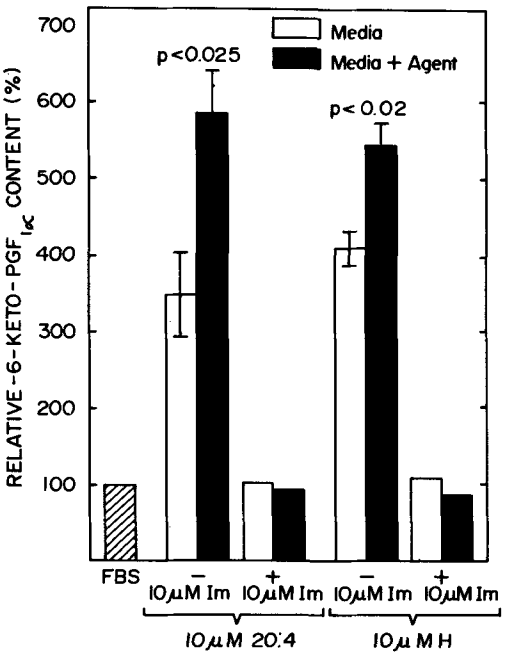

FIG. 3. Effect of either 20:4(n-6) or hydralazine (H) with or without indomethacin on PGI_2 biosynthesis in growing cultures. Cells were cloned either in 10% or 20% FBS. Data are expressed as relative 6-keto-$PGF_{1}\alpha$ content compared to FBS. Media with 20% FBS contained 3.59 ± 0.14 pmol/culture (mean $\pm$ SEM for 24 samples). Vertical lines show SEM for data from 3 different primary cultures. Significant differences are noted in the figure.

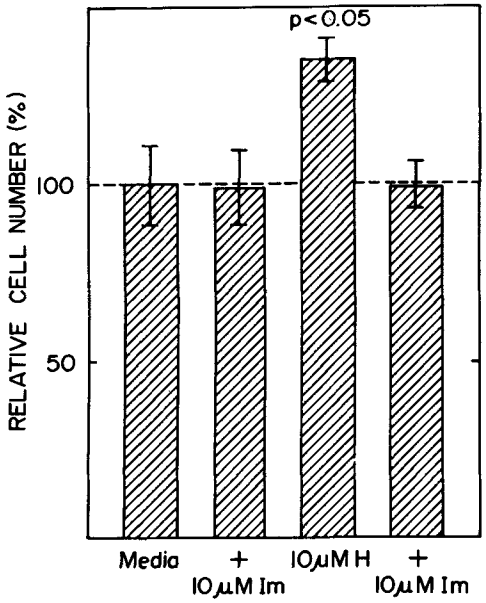

FIG. 4. Effect of 20:4(n-6) and indomethacin (Im) on cell proliferation. Cells were cloned for 8 days in Costar tissue culture dishes containing media alone or media supplemented with 20:4(n-6) and/or indomethacin. Each group contained 8 plates. Data are expressed as relative cell number compared to control (media alone). Vertical lines show SEM. Significant differences are noted in the figure.

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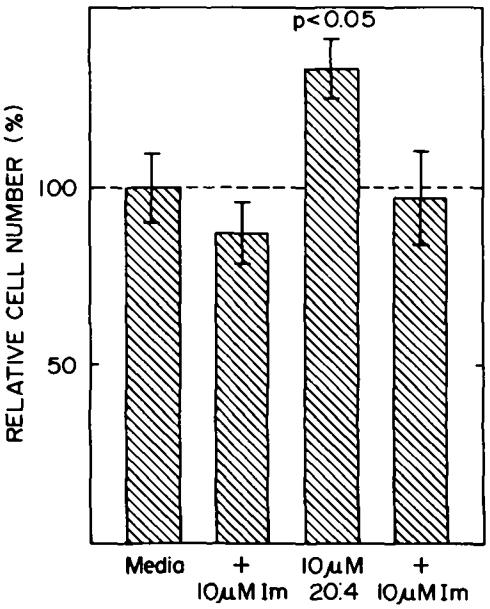

FIG. 5. Effect of hydralazine (H) and indomethacin (Im) on cell proliferation. Cells were cloned for 8 days in Costar tissue culture dishes containing media alone or media supplemented with hydralazine and/or indomethacin. Each group contained 8 plates. Data are expressed as relative cell number compared to control (media alone). Vertical lines show SEM. Significant differences are noted in the figure.

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