BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **235,** 418–423 (1997)
ARTICLE NO. RC976799

Arachidonic Acid Stimulates Prostate Cancer Cell Growth: Critical Role of 5-Lipoxygenase

Jagadananda Ghosh and Charles E. Myers
University of Virginia Cancer Center, P. O. Box 334, Charlottesville, Virginia 22908

Received May 15, 1997

Arachidonic acid (5,8,11,14-eicosatetraenoic acid), a member of the omega-6 poly-unsaturated fatty acids, was found to be an effective stimulator of human prostate cancer cell growth *in vitro* at micromolar concentrations. Selective blockade of the different metabolic pathways of arachidonic acid (e.g. ibuprofen for cyclooxygenase, SKF-525A for cytochrome P-450, baicalein and BHPP for 12-lipoxygenase, AA861 and MK886 for 5-lipoxygenase, etc.) revealed that the growth stimulatory effect of arachidonic acid is inhibited by the 5-lipoxygenase specific inhibitors, AA861 and MK886, but not by others. Addition of the eicosatetraenoid products of 5-lipoxygenase (5-HETEs) showed stimulation of prostate cancer cell growth similar to that of arachidonic acid, whereas the leukotrienes were ineffective. Moreover, the 5-series of eicosatetraenoids could reverse the growth inhibitory effect of MK886. Finally, prostate cancer cells fed with arachidonic acid showed a dramatic increase in the production of 5-HETEs which is effectively blocked by MK886. These experimental observations suggest that arachidonic acid needs to be metabolized through the 5-lipoxygenase pathway to produce 5-HETE series of eicosatetraenoids for its growth stimulatory effects on human prostate cancer cells.

Prostate cancer is one of the most common forms of human malignancy, affecting greater than 80% of men over the age of 70. Based upon autopsy studies, current estimates are that more than 20 million men in the United States have occult prostate cancer. The development of metastatic prostate cancer is much less common, with 40-50,000 men presenting with this complication in any given year. The metastatic, lethal form of prostate cancer is characterized by more rapid growth as well as the ability to spread to tissues distant from the prostate. Localized prostate cancers that are curable by surgery or radiation therapy exhibit tumor doubling times of between 2 and 4 years. In contrast, prostate cancer that is metastatic at presentation or recurs after radiation therapy typically will show tumor doubling time of 3-4 months. In extreme cases, tumor doubling time can be as short as 10-14 days.

The transition of prostate cancer from a slow growing, localized disease to a rapidly growing metastatic tumor appears to be influenced by environmental factors. While the incidence of localized prostate cancer is the same in all of the developed countries, the risk of developing metastatic disease is more than 90% lower in Japan, Hong Kong and Singapore than it is in the United States and Europe. The two factors most commonly implicated in this difference are circulating androgen levels and dietary fat, although the epidemiological evidence for both remains controversial (1, 2).

There are now multiple reports supporting a role for arachidonic acid in the proliferation of prostate cancer cells (3-5). In radical prostatectomy specimens, arachidonic acid turnover is 10 times greater in the tumor as compared to surrounding uninvolved prostate tissue. In nude mice, diets high in corn oil, a fat rich in the arachidonic acid precursor linoleic acid, markedly stimulate the growth of human prostate cancer xenografts (6). Epidermal growth factor and the related cytokines, TGF-α and amphiregulin, have been implicated in both androgen dependent and androgen independent proliferation of prostate cancer cells (7-10). These cytokines all act through the EGF receptor. Release of arachidonic acid from membrane phospholipids in response to EGF receptor activation is common to many different normal and malignant tissues (11-13). Furthermore, arachidonic acid release appears to be an obligatory step in the mitogenesis as well as motility and cytoskeletal rearrangement induced by EGF and related growth factor-receptor activation (12, 14).

Arachidonic acid can directly modulate intracellular targets like protein kinase C and ras-GAP (15-17). In addition, arachidonic acid can undergo metabolic conversion to a wide range of eicosanoids including prostaglandins, thromboxanes and leukotrienes (18, 19). Members of each of these three eicosanoid families have been described as mitogens in one or more cell

0006-291X/97 $25.00

types particularly of tumor origin (19, 20). In addition, many of the eicosanoids were observed to suppress immune surveillance (21). As a result, inhibition of arachidonic acid metabolism has become an attractive new target area for chemotherapeutic manipulation of various types of cancer (22).

With this background, we have examined in greater detail the role of arachidonic acid in the regulation of human prostate cancer cell growth. Our results indicate that arachidonic acid is a potent mitogen for human prostate cancer cells and that these cells need to metabolize arachidonic acid via the 5-lipoxygenase pathway to produce 5-HETE series of eicosatetraenoids. These eicosatetraenoids are also sufficient to support proliferation of prostate cancer cells and could reverse the effect of 5-lipoxygenase inhibitors. Furthermore, very recently, we have found that human prostate cancer cells rapidly undergo apoptosis when they are deprived of arachidonic acid or when the subsequent metabolism of arachidonic acid by the 5-lipoxygenase pathway is interrupted (J. Ghosh and Charles E. Myers; Manuscript in Preparation). These findings provide a mechanism by which dietary fat intake might influence the natural history of prostate cancer.

MATERIALS AND METHODS

Materials. Arachidonic acid, fatty acid-free bovine serum albumin (BSA) and nor-dihydro guaiaretic acid (NDGA) were purchased from Sigma Chemical Company (St. Louis, MO). Eicosanoids were purchased from Cayman Chemical Company (Ann Arbor, MI) or Cascade Biochem Ltd (Berkshire, UK). Ibuprofen, baicalein, AA861 and SKF-525A were purchased from Biomol (Plymouth Meeting, PA). BHPP was a kind gift from John Conway of Biomide Corporation (Grosse Pointe Farms, MI). MK886 is a compound from Merck-Frosst, Canada.

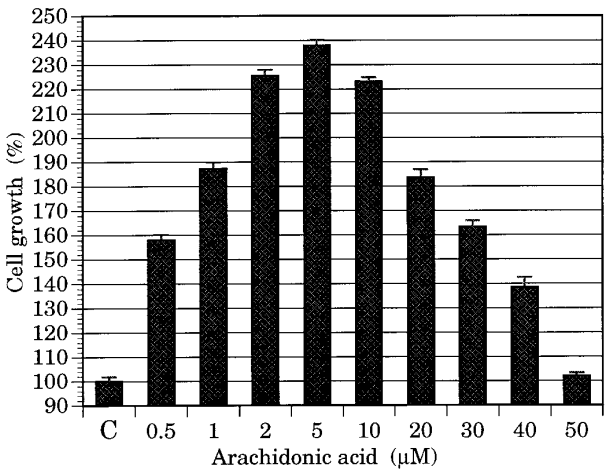

FIG. 1. Modulation of prostate cancer cell growth by arachidonic acid. PC3 cells (2500 per well) were plated overnight in RPMI medium supplemented with 0.5% FBS in 96 well tissue culture plates. Arachidonic acid was then added pre-complexed with lipid-free BSA (final concentration of BSA, 5 μM) and the plates were further incubated for 72 hours. Control cells were grown in the plating medium containing 5 μM lipid-free BSA and the solvent (0.02% DMSO). Cell growth was measured by MTS/PMS Cell Titer Assay as described in the "Methods" section. Results shown here are representative of several similar experiments. Each data point represents the mean (n=8) ± standard error.

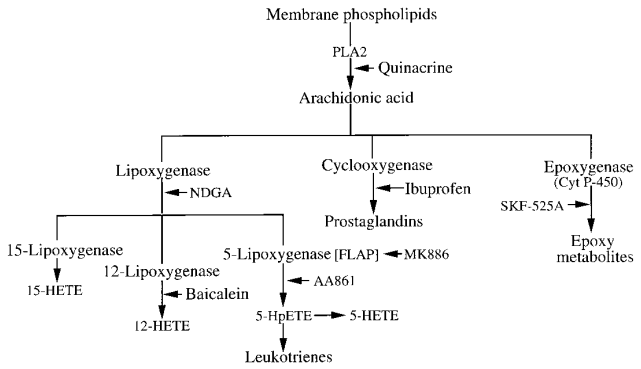

FIG. 2. Schematic representation of the various enzymes and inhibitors involved in major pathways of arachidonic acid metabolism (for explanation see text).

Cell culture. Androgen independent (PC3) and androgen dependent (LNCaP) prostate cancer cell lines were obtained from American Type Culture Collection (Rockville, MD). Cells were grown in RPMI-1640 supplemented with 10% FBS plus 100 U/ml of penicillin and 100 μg/ml of streptomycin. Cultures were maintained at 37°C in humidified CO_2-incubator. Cells were fed with fresh medium every third day and passaged routinely at a confluence of ~80 %.

Cell growth assay. Cell growth was measured using the MTS/PMS Cell-Titer 96 AQ assay (Promega Corporation). Cells (~2500/well) in 100 μl RPMI medium were allowed to plate overnight at 37°C in the incubator in 96 well plates (Falcon). On day-2 experimental agents were added with the proper controls and the plates were further incubated for 72 hours. Fatty acids and eicosanoids were pre-complexed with lipid-free bovine serum albumin (BSA) before addition to the medium. Final concentration of BSA was maintained at 5 μM throughout the experiments. Control cells were treated with media containing BSA only. Inhibitors of eicosanoid synthesis were initially solubilized in DMSO and further diluted in serum-free RPMI to get desired concentrations. The final concentration of DMSO in culture medium was 0.01-0.05%. Control culture wells received equivalent amounts of the vehicle only. At the end of culture periods, 20 μl aliquots of a mixture of MTS and PMS were added and the plates were incubated for 2 hours at 37°C in the incubator. Color of the soluble brown formazan product was measured using a TiterTek microplate reader (ICN Biomedicals).

Radioimmunoassay of 5-hydroxyeicosatetraenoids (5-HETEs). PC3 prostate cancer cells (6×10^5) were plated overnight in 60 mm diameter Petri dishes in RPMI medium supplemented with 0.5% FBS. Before the experiment the medium was replaced with fresh 2 ml RPMI medium and the cells were treated with 5 μM arachidonic acid precomplexed with lipid-free BSA. The final concentration of BSA was 5 μM throughout the experiments. MK886 was primarily dissolved in DMSO and further diluted with RPMI medium before addition to the culture. Control cells were treated with the plating medium containing 0.01% DMSO and 5 μM BSA. At the end of incubation period aliquots of the culture supernatants were taken and the amounts of 5-HETEs were measured by radioimmunoassay (Perseptive Diagnostics, Framingham, MA).

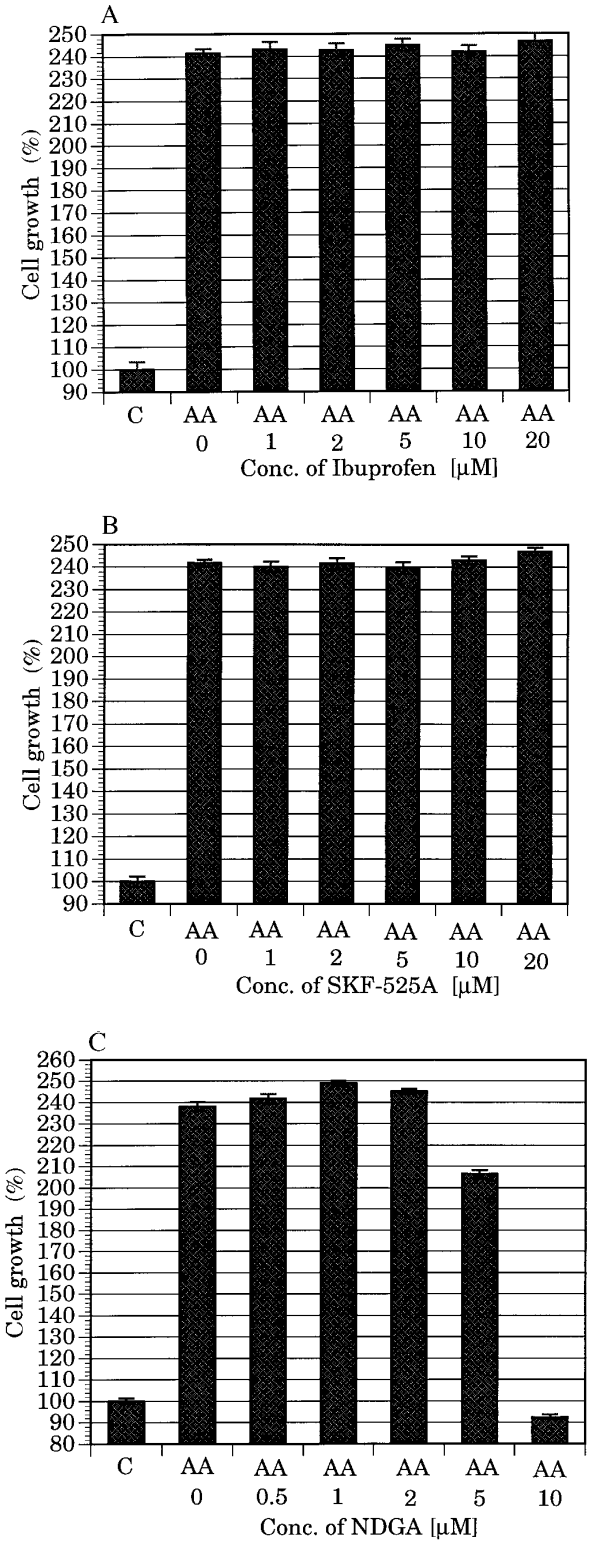

FIG. 3. Effect of various metabolic inhibitors on the growth stimulatory effect of arachidonic acid. Cells were plated as described "in figure 1" and treated with 5 μM of arachidonic acid (AA) in absence or presence of varying doses of ibuprofen (cyclooxygenase inhibitor), SKF-525A (epoxygenase inhibitor) or NDGA (lipoxygenase inhibitor). Control cells were treated with the plating medium containing the solvent (0.02% DMSO) and 5 μM BSA. Cells were further incubated for 72 hours and the cell growth was measured by MTS/PMS Cell Titer Assay. Data presented as the mean (n=8) ± standard error.

RESULTS

Arachidonic Acid Stimulates Human Prostate Cancer Cell Growth

Based on the reports of epidemiological studies and animal models that high fat diet might have a positive link with prostate cancer growth, we were interested to see whether arachidonic acid, itself, can support prostate cancer cell growth *in vitro.* Results, depicted in figure 1, clearly show that arachidonic acid, the metabolic product of PLA2, is indeed a powerful stimulus of prostate cancer cell growth at micromolar concentrations. Similar reproducible, dose dependent, stimulation of cell growth by arachidonic acid was observed in the hormone-dependent LNCaP prostate cancer cells (not shown). Maximum growth stimulation was observed at 5-10 μM of exogenous arachidonic acid, while higher concentrations of arachidonic acid inhibited growth of the prostate cancer cells.

Metabolism of Arachidonic Acid Is Critical for Its Stimulation of Prostate Cancer Cell Growth

Arachidonic acid can be metabolized to produce eicosanoids through a variety of metabolic pathways (Figure 2). In addition, arachidonic acid can act directly to simulate the activity of PKC. We next examined whether the mitogenic activity of arachidonic acid was the result of direct interaction with molecular targets or required further metabolism to one of the eicosanoids. Ibuprofen and SKF-525A, two specific inhibitors of cyclooxygenase and cytochrome P-450 mediated-epoxygenase pathways respectively, were found to have no effect on cell growth stimulation by arachidonic acid. On the other hand, NDGA, a general inhibitor of the lipoxygenase pathways, completely inhibited arachidonic acid-stimulated growth of prostate cancer cells, suggesting that the mitogenic activity of arachidonic acid in prostate cancer cells might involve metabolic conversion by one of the lipoxygenases (Figure 3 A-C).

Baicalein, a select inhibitor of the 12-lipoxygenase pathway (ID_{50} 0.12 μM), did not show any effect on arachidonic acid-stimulated growth of prostate cancer cells (Figure 4 A). A similar lack of effect was observed with BHPP, another select inhibitor of the 12-lipoxygenase (not shown). In contrast, AA861, a specific inhibitor of 5-lipoxygenase, completely inhibited arachidonic acid-stimulated growth of PC3 prostate cancer cells (Figure 4 B). Moreover, MK886, an inhibitor of 5-lipoxygenase activating protein (FLAP), was found

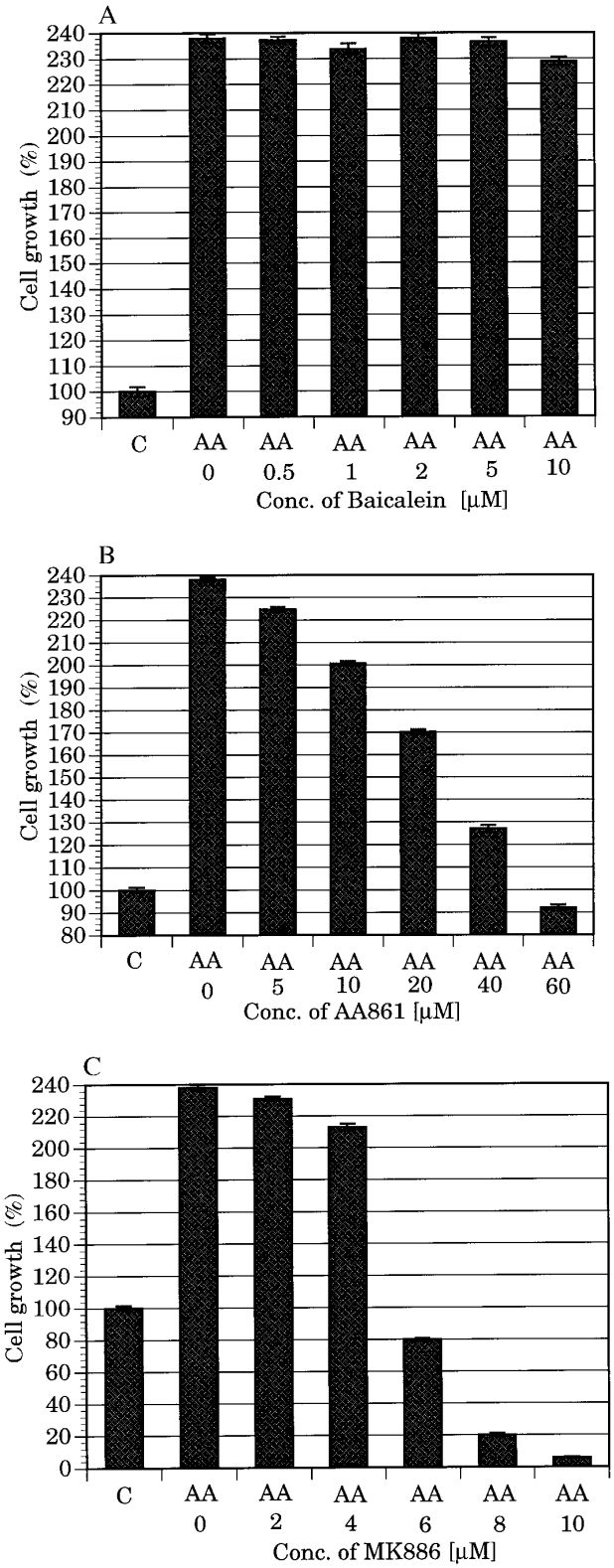

FIG. 4. Inhibitors of 5-lipoxygenase but not 12-lipoxygenase block arachidonic acid stimulated growth of prostate cancer cells. PC3 prostate cancer cells were plated overnight in RPMI medium supplemented with 0.5% FBS. Cells were then treated with 5 μM arachidonic acid (AA) in the absence or presence of varying doses of Baicalein (A), AA861 (B), or MK886 (C). Control cells were treated with the plating medium containing 5 μM BSA and the solvent (0.02% DMSO). Cultures were incubated further for 72 hours and the cell growth was measured by MTS/PMS Cell Titer Assay. Data represents the mean (n=8) ± standard error.

TABLE 1

Mitogenic Activity of 5-Lipoxygenase Products of Arachidonic Acid Metabolism

Compound	Maximal growth stimulation (%)	Concentration
Arachidonic acid	140	10 μM
5-HpETE	50	5 μM
5-HETE	50	10 μM
5-HETE lactone	90	10 μM
5-oxoETE	110	10 μM
LTB4	0	10 μM
LTC4	0	10 μM
LTD4	0	10 μM

PC3 prostate cancer cells were plated overnight in RPMI medium supplemented with 0.5% FBS. Experimental agents (all pre-complexed with lipid-free BSA) were then added and the plates were further incubated for 72 hours. Control cells were cultured in plating medium containing 5 μM lipid-free BSA and the solvent (0.02% DMSO). Cell growth was measured by MTS/PMS Cell Titer Assay. Data reported here represent percent increase in cell growth over control (n=8).

to be a strong inhibitor of arachidonic acid-stimulated prostate cancer cell growth (4 C). These experimental observations suggest that metabolic conversion through the 5-lipoxygenase pathway is an important step in the regulation of arachidonic acid stimulated growth of prostate cancer cells.

Which Eicosanoid Products of 5-Lipoxygenase Stimulate Prostate Cancer Cell Growth?

Both leukotrienes and hydroxyeicosatetraenoids are produced from arachidonic acid by the action of 5-lipoxygenase. In order to better identify the specific 5-lipoxygenase products able to support the proliferation of prostate cancer cells, we next examined which of these eicosanoids were capable of stimulating the growth of PC3 cells under serum reduced conditions. Results are depicted in Table 1 and show that 5-HpETE and 5-HETE and its metabolites, but not the leukotrienes, are able to support the proliferation of PC3 cells suggesting that the eicosatetraenoids are active 5-lipoxygenase metabolites in these cells.

Next we were interested to see whether the eicosatetraenoid products of 5-lipoxygenase could reverse the inhibitory effect of MK886. Results are depicted in fig-

ure 5, which shows that 5-oxoETE could effectively reverse the growth inhibition by MK886. Similar effects were also seen with 5-HETE lactone (not shown). These observations confirm that the 5-series of eicosatetraenoids are active metabolites in human prostate cancer cells and that the growth inhibition by MK886 is the result of specific inhibition of arachidonic acid metabolism by the 5-lipoxygenase.

Prostate Cancer Cells Produce 5-HETEs from Arachidonic Acid

Experiments with inhibitors and metabolites suggested that prostate cancer cells produce 5-HETE series of eicosanoids from arachidonic acid. We next tried to get a more definitive answer by measuring 5-HETEs directly after feeding the cells with exogenous arachidonic acid. Figure 6 shows that arachidonic acid addition resulted in rapid increase in the production of 5-HETEs, which is effectively blocked by the 5-lipoxygenase inhibitor, MK886. Blockade of arachidonic acid-stimulated growth by 5-lipoxygenase inhibitors, reversal of the inhibition by 5-lipoxygenase metabolites, and a rapid increase in the production of 5-HETE series of eicosanoids from arachidonic acid altogether suggest that the growth stimulatory effect of arachidonic acid is presumably mediated by its metabolic conversion through the 5-lipoxygenase pathway.

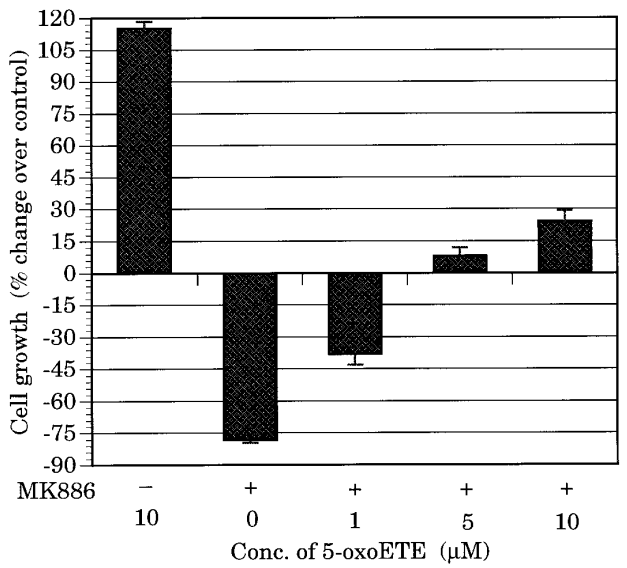

FIG. 5. Reversal of the effect of 5-lipoxygenase inhibition by 5-oxoETE. PC3 prostate cancer cells were plated in RPMI medium supplemented with 0.5% FBS in 96 well tissue culture plates. Cells were then treated with varying doses of 5-oxoETE and 6 μM of the 5-lipoxygenase inhibitor, MK886. Cells were incubated further for 72 hours. Control cells were treated with the plating media containing the solvent (0.02% DMSO) and 5 μM BSA. At the end of incubation period cell growth was measured by MTS/PMS Cell Titer Assay. The results are presented as the mean (n=8) ± standard error.

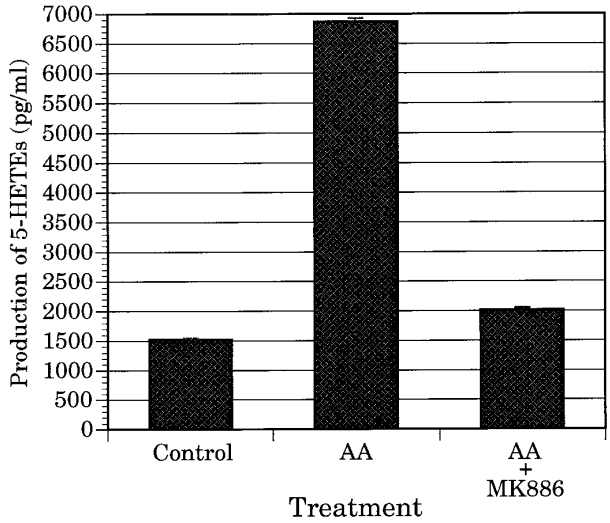

FIG. 6. Stimulation of 5-HETE production by arachidonic acid. PC3 cells were plated as described before and treated with 5 μM of arachidonic acid in the absence or presence of 10 μM of MK886 for 2 hours. Control cells were treated with the plating medium containing the solvent (0.02% DMSO) and 5 μM BSA. Amount of 5-HETE production was measured by radioimmunoassay (RIA). Results shown as the mean (n=2) ± SD.

DISCUSSION

Though epidemiological studies and experiments with animal models support the hypothesis that dietary fat might play a role as a risk factor for the growth and development of prostate cancer, biochemical evidence to substantiate a link between components of dietary fat or their metabolites and mitogenesis of prostate cancer cells has been lacking. Arachidonic acid and its precursor, linoleic acid, are major components of animal fat and many vegetable oils used in regions where prostate cancer is common. As we observed, arachidonic acid can stimulate the mitogenesis of human prostate cancer cells *in vitro*, our findings suggest that a high fat diet rich in arachidonic acid or its precursor n-6 fatty acids can provide a powerful stimulus to the proliferation of human prostate cancer cells.

By using specific inhibitors of different pathways we determined that metabolic conversion of arachidonic acid through 5-lipoxygenase is required for its growth stimulatory effect on human prostate cancer cells. Moreover, of the two different classes of 5-lipoxygenase metabolites, only the eicosatetraenoids were found to stimulate prostate cancer cell growth and reverse the inhibitory effects of 5-lipoxygenase inhibitors, whereas the leukotrienes were of no effect. These results are consistent with the production of 5-HETE series of eicosatetraenoids by these cells from arachidonic acid. Our experimental observations demonstrated that indeed the prostate cancer cells

produce 5-hydroxyeicosatetranoids and this process is dramatically enhanced by arachidonic acid addition. Furthermore, production of 5-HETE series of eicosatetraenoids was blocked by the 5-lipoxygenase inhibitor, MK886. These findings prove that for prostate cancer cells the 5-HETE series of eicosatetraenoids mediate arachidonic acid dependent mitogenesis. Both prostaglandins and leukotrienes are known to act via specific membrane bound receptors (23, 24). Whether the 5-HETE series of eicosatetraenoids exert their biological function by regulating the activity of protein kinase C or they signal through their own G-protein coupled receptors is an interesting area of future study. Moreover, like 12- and 15-HETEs, 5-HETE might inhibit the activity of diacylglycerol kinase and promote the accumulation of diacylglycerol (25).

5-Lipoxygenase knockout mouse strains have been developed. These mice grow normally and give birth to fertile offsprings, indicating that the function of 5-lipoxygenase is not an absolute requirement for most body tissues (26, 27). Moreover, drugs that block 5-lipoxygenase were found to be effective in the treatment of asthma and are well tolerated in man and a number of experimental animals. In this context, our present findings open up an attractive new target area to hinder the growth promoting activity of dietary arachidonic acid and its precursors in human prostate cancer.

ACKNOWLEDGMENTS

This work was partially supported by the CaPCure Foundation, a donation by Lynch family and by an NCI Development Grant in Prostate Cancer, R21-CA 69848.

REFERENCES

1. Gann, P. H., Hennekens, C. H., Sacks, F. M., Grodstein, F., Giovannucci, E. L., and Stampfer, M. J. (1994) *J. Natl. Cancer Inst.* **86,** 281–286.
2. Brawley, O. W., and Thompson, I. M. (1994) *Urology* **43,** 594–599.
3. Chaudry, A. A., Wahle, K. W., McClinton, S., and Moffat, L. E. (1994) *Int. J. Cancer* **57,** 176–180.
4. Dahiya, R., Yoon, W. H., Boyle, B., Schoenberg, S., Yen, T. S., and Narayan, P. (1992) *Biochem. Int.* **27,** 567–577.
5. Anderson, K. M., Wygodny, J. B., Ondrey, F., and Harris, J. (1988) *Prostate* **12,** 3–12.
6. Wang, Y., Corr, J. G., Thaler, H. T., Tao, Y., Fair, W. R., and Heston, W. D. (1995) *J. Natl. Cancer Inst.* **87,** 1456–1462.
7. Cohen, D. W., Simak, R., Fair, W. R., Melamed, J., Scher, H. I., and Cordon, C. C. (1994) *J. Urol.* **152,** 2120–2124.
8. Connolly, J. M., and Rose, D. P. (1990) *Prostate* **16,** 209–218.
9. MacDonald, A., and Habib, F. K. (1992) *Br. J. Cancer* **65,** 177–182.
10. Schuurmans, A. L., Bolt, J., Voorhorst, M. M., Blankenstein, R. A., and Mulder, E. (1988) *Int. J. Cancer* **42,** 917–922.
11. Glasgow, W. C., Afshari, C. A., Barrett, J. C., and Eling, T. E. (1992) *J. Biol. Chem.* **267,** 10771–10779.
12. Peppelenbosch, M. P., Tertoolen, L. G., Hage, W. J., and de, L. S. (1993) *Cell* **74,** 565–575.
13. Liu, P., Wen, M., Sun, L., and Hayashi, J. (1995) *Biochem. J.* **308,** 399–404.
14. Dethlefsen, S. M., Shepro, D., and D'Amore, P. A. (1994) *Exp. Cell Res.* **212,** 262–273.
15. Khan, W. A., Blobe, G. C., and Hannun, Y. A. (1995) *Cell. Signalling* **7,** 171–184.
16. Schachter, J. B., Lester, D. S., and Alkon, D. L. (1996) *Biochim. Biophys. Acta* **1291,** 167–176.
17. Bollag, G., and McCormick, F. (1991) *Nature* **351,** 576–579.
18. Samuelsson, B., Dahlen, S. E., Lindgren, J. A., Rouzer, C. A., and Serhan, C. N. (1987) *Science* **237,** 1171–1176.
19. Needleman, P., Turk, J., Jakschik, B. A., Morrison, A. R., and Lefkowith, J. B. (1986) *Ann. Rev. Biochem.* **55,** 69–102.
20. Tang, D. G., Renaud, C., Stojakovic, S., Dilgio, C. A., Porter, A., and Honn, K. V. (1995) *Biochem. Biophys. Res. Commun.* **211,** 462–468.
21. Young, M. R. (1994) *Cancer Met. Rev.* **13,** 337–348.
22. Ara, G., and Teicher, B. A. (1996) *Prostaglandins, leukotrienes and Essential Fatty Acids* **54,** 3–16.
23. Haluska, P. V., Mais, D. E., Mayeux, P. R., and Morinelli, T. A. (1989) *Ann. Rev. Pharmacol. Toxicol.* **10,** 213–239.
24. Smith, W. L. (1989) *Biochem. J.* **259,** 315–324.
25. Setty, B. N. Y., Graeber, J. E., and Stuart, M. J. (1987) *J. Biol. Chem.* **262,** 17613–17622.
26. Chen, X., Sheller, J., Johnson, E., and Funk, C. D. (1994) *Nature* **372,** 179–182.
27. Funk, C. D., Kurre, U., and Griffis, G. (1994) *Ann. N. Y. Acad. Sci.* **714,** 253–258.