J. Food Sci. Nutr.
Vol. 4, No. 3, p. 203～208 (1999)

Effects of Polyunsaturated Fatty Acids on Intestinal Cell Proliferation

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Abstract

The effect of the polyunsaturated fatty acids, linoleic acid (LA), arachidonic acid (AA) and conjugated dienoic linoleic acid (CLA) on IEC-6 cells (rat intestinal cell) proliferation and cell transduction have been determined *in vitro*. IEC-6 cells proliferation was assessed by cell growth and [^{3}H]-thymidine incorporation analysis. At 10 μM concentration, the proliferation of cells supplemented with AA or LA was significantly higher than that of CLA. [^{3}H]-thymidine uptake showed the same results. LA and AA increased [^{3}H]-thymidine uptake more than CLA. The stimulatory effect of LA or AA was even more pronounced in the presence of IGF. Both cell number analysis and [^{3}H]-thymidine incorporation revealed that IEC-6 cell proliferation was influenced differently by exogenous free fatty acids, in which AA or LA stimulated IEC-6 cell proliferation and CLA inhibited it. Tyrosine phosphorylation provides a key switch to regulate cellular activity in response to extracellular stimuli. At 20 μM and 10 μM, AA with IGF-1 stimulated protein tyrosine phosphorylation in IEC-6 cells, but LA's impact was less than that of AA. CLA and CLA with IGF-1 inhibited protein tyrosine phosphorylation in IEC-6 cells. These results suggest there is a possible correlation between cell proliferation and IGF receptor tyrosine kinase activity driven by AA.

Key words: arachidonic acid, linoleic acid, conjugated dienoic linoleic acid, cell growth, tyrosine phosphorylation

INTRODUCTION

For more than half a century, evidence has been accumulated that shows a relationship of both level and type of dietary fat to the development of cancer (1-5). In general, polyunsaturated fatty acids (PUFAs) are more positively associated with tumorigenesis than saturated fatty acids (6-8). PUFAs of the n-6 class usually correlates with enhanced tumorigenesis in several organs. In contrast, n-3 class appears to be associated with inhibition of carcinogenesis (9-11).

The n-6 PUFAs, primarily linoleic acid and archidonic acid, were reported to promote tumorigenesis and tumor cell proliferation directly and indirectly via increased synthesis of cyclooxygenase- and lipoxygenase-catalyzed products (10,12,13). Arachidonic acid is one of the major PUFAs present in the mammalian cell membrane. Arachidonic acid and its metabolites have been identified as a novel group of intracellular second messengers (14). Other studies suggest that this group regulated activities of enzymes which were involved in signal transduction such as protein kinase (15). Exogenous arachidonic acid could alter fatty acid profiles of membrane phospholipid (16,17). So cell proliferation is influenced by changes in phospholipid fatty acid composition of cell membranes. Proliferation of intestinal cells is considered to be a significant factor in the development of colon cancer (18-20).

Conjugated dienoic linoleate (CLA) refers to a group of linoleate (cis9,c12-octadecadienoate) derivatives that exhibit the possibility of several positional and geometric isomers (21). CLA has been found in triglycerides, lipoptotein and cell membrane phospholipids in several tissues of rodents, rabbits and human. In foods, CLA is the highest in ruminant meats and is found in smaller amounts in poultry and eggs. Dairy products contain considerable amounts of CLA. Vegetable fats are poorer sources of CLA (21,22). CLA is a unique PUFA that has been shown to inhibit carcinogenesis in several experimental animal models. Because CLA is rapidly incorporated into cell membrane phospholipids it may inhibit carcinogenesis by modulating several cellular events that are mediated in part by the lipid milieu of the plasma membrane (22,23).

The mucosa of the small intestine represents a distinctive population of cells that perpetually regenerate themselves in an effort to balance cell loss through exfoliation by the process of cellular proliferation and growth (24,25). Multiple factors are involved in the regulation of intestinal epithelial cell proliferation and differentiation. These factors include the general nutritional status of animal, luminal nutrients, endocrine factors, paracrine factors and cell : cell interaction (24,25). The insulin like growth factors (IGF-1, IGF-II) are polypeptides with structural similarity to proinsulin that stimulate cell proliferation by endocrine, paracrine and autocrine mechanism (26). Insulin and the insulin like growth factors, IGF-1, IGF-II, stimulate the growth of IEC-6 cells, an intestinal cell line derived from jejunal crypts of a rat (27). Among these three growth factors, IGF-1 was the most effective. The initial event in the metabolic action of IGFs on target cells appears to begin by binding to specific receptors on the plasma membrane. The IGF-1 receptor is the alpha2 beta2 transmembrane glycoprotein that contains tyrosine protein kinase domains in the cytoplasmic portion of the beta subunit (26,28). IGF binding results in receptor dimerization, thereby activating the intrinsic receptor tyrosine kinase activity, causing its autophos-

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phorylation and signal propagation to downstream substrates (29). Tyrosine phosphorylation provides a key switch to regulate cellular activity in response to extracellular stimuli (30). Although IGF-1 is the major mediator of growth hormone's action on somatic growth, nutritional status of an organism is a critical regulator of IGF-1 (31). So IGF-mediated signal transduction may be modulated by fatty acids.

The objectives of this study is to observe the effects of AA, LA, CLA on cell proliferation and on IGF receptor autophosphorylation which provides a key switch to regulate cellular activity in response to extracellular stimuli.

MATERIALS AND METHODS

Cell culture and culture conditions

In this study we examined IEC-6 cells, an intestinal cell line derived from rat jejunal crypts (27). IEC-6 cells were maintained in Dulbecco's modified eagles medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% L-gutamine, and incubated at 37°C in a humidified atmosphere with 5% CO_2. Stock cultures were divided weekly and routinely tested for mycoplasm contamination.

Stock solution of fatty acids (linoleic acid, arachidonic acid, conjugated dienoic linoleate and stearic acid) were prepared in 99% ethanol and stored at -70°C under nitrogen. After diluting the stocks with 0.1% bovine serum albumin (BSA), they were stored at -20°C. After addition of fatty acids, the medium was warmed for 1 hr at 37°C, before addition to cell cultures.

Dose response

Cells were plated at a density of 3×10^4 cells/ml in 35 mm dishes in DMEM containing 10% FBS for 1 ~ 2 days. After cell numbers reached $15 \sim 20 \times 10^4$, cells were washed with phosphate buffered saline (PBS), then incubated in serum-free DMEM with 100 µM, 50 µM, 20 µM, 10 µM, 5 µM, 1 µM, 0.5 µM, free fatty acids (arachidonic acid, linoleic acid, conjugated dienoic linoleate, stearic acid) plus 0.1% BSA and 10% FBS. After 72 hours, cell growth was determined by trypsinizing and cell count by a hemocytometer.

Cell growth

Cell growth curve

After determining optimum concentration, cells were plated at a density of 3×10^4 cells/ml in 35 mm dishes in DMEM containing 10% FBS for 1 day. Cells were washed with PBS, then incubated in serum free DMEM for 2 hr. The cells were then exposed to serum-free DMEM with determined concentration of fatty acids (arachidonic acid, linoleic acid, conjugated dienoic linoleate, stearic acid). As a control, cells were cultured in DMEM plus 0.1% BSA throughout the treatment period. The treatment media were changed everyday during the treatment period. Cell growth was determined by trypsinizing and cell count by a hemocytometer every day for 5 days.

[^{3}H] thymidine incorporation

Cells were plated at a density of 3×10^4 cells/ml in 35 mm dishes for 1 day. Cells were washed with PBS, then incubated in serum-free DMEM for 2 hr to remove bound growth factors. The cells were, then, exposed to serum free DMEM with or without 10 µM concentration of free fatty acids (arachidonic acid, linoleic acid, conjugated dienoic linoleate and stearic acid). As a control, cells were cultured in DMEM plus 0.1% BSA throughout the treatment period. The treatment media were changed everyday during the treatment period. From day - 0 after washing 3 times with PBS, cells were incubated with (^{3}H) thymidine (0.05 uCi/ml) for 4 h in the continued absence or presence of fatty acid treatment everyday throughout the 4 day treatment period. Cells were washed three times with PBS, and lysed in 0.24 ml 1 N NaOH. 75 µl of the lysates were counted in a liquid scitillation spectrometer.

Tyrosine phosphorylation

To test the effect of LA, AA, CLA on tyrosine phosphorylation, IEC-6 cells were plated at a density of 3×10^4 cells/ml in 100 mm dishes. After 80% confluency, cells were washed twice with PBS, then exposed to serum-free DMEM with or without determined concentration of free fatty acids (arachidonic acid, linoleic acid, conjugated dienoic linoliate, steric acid) plus 0.1% BSA for 16 hr or 1 day. After incubation, media were aspirated then added to 0.1% BAS with or without IGF. After 3 min, cells were rinsed twice with cold PBS. Cells were then thawed in 250 µl for lysis buffer. Cells lysates were cleared by centrifugation at 14,000 rpm for 10 min in a microcentrifuge. The protein content of cell lysates was determined using Bio-rads's reagent kit. Cell lysates containing amounts of protein (20 µg/lane) from each condition were separated by SDS polyacryamide gel electrophoresis and transferred electrophoretically to nitocellulose membranes. After treating primary and secondary antibody, fluorescent solution was added, and then developed.

Statistical analysis

Data are presented as means ± standard deviation. Statistical evaluation of the results are carried by ANOVA, treatment effects are analysed by Duncan's test.

RESULTS AND DISCUSSION

Dose response

While linoleic acid (LA) and archidonic acid (AA) of the n-6 PUFAs, promote tumorigenesis and tumor cell proliferation, conjugated dienoic linoleic acid (CLA) is a unique PUFA that has been shown to inhibit carcinogenesis in several experimental animal models (10,23); the effects of different concentrations of these free fatty acids on the cell growth of IEC-6 cells for 72 hr were investigated. At higher concentration than 30 µM LA and AA inhibited cell proliferation, so at 30 µM, 20 µM, 10 µM, 7 µM of the concentrations AA · LA · SA · CLA were tested. As shown in Fig. 1, the optimum concentration of AA, LA, CLA and SA on IEC-6 cell growth for 72 hr was between 10 µM and 7 µM, AA and LA stimulated IEC-6 cell growth more than SA and CLA. In con-

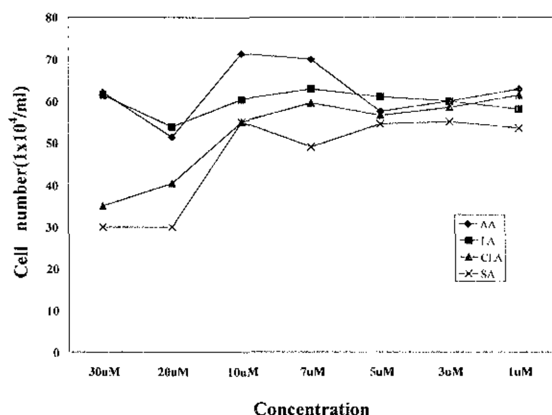

Fig. 1. Effects of various concentration of arachidonic acid (AA), linoleic acid (LA), conjugated linoleic acid (CLA) and stearic acid (SA) on the growth of IEC-6 cells for 72 hours.

trast the proliferation stimulated by all these fatty acids at concentration<7 µM for 72 hr yield no significant differences.

Hussy's study (7) showed the optimal concentration of both LA and AA required for Mac26 and Mac13 (murine colon adenocarcinomas) growth stimulation in vitro lay between 18 and 33 µM, and growth inhibition was observed at higher concentrations. Thus, these PUFAs appear to have a dual effect on tumor cell growth in vitro. The growth inhibition observed at higher concentration is probably explained by the toxicity of free fatty acids. They used the fatty acids which were complexed with equal weights of bovine serum albumin, neutralised with sodium bicarbonate. Mollerup and Haugen (32) & Ghosh and Myers (33) performed a dose response using AA dissolved ethanol : AA stimulated cell proliferation at concentration<10 µM in Mollerup's study, 2~10 µM at Ghosh's study. As in these studies there were differences of optimal concentration of AA and LA according to cell lines and the way fatty acids were prepared.

Cell growth

As a result of these dose responses the optimum concentration of AA, LA, CLA and SA on IEC-6 cell growth for 72 hr was between 10 µM and 7 µM. In order to compare the effects of AA, LA, CLA and SA at 10 µM on cell proliferation in IEC-6 cell lines, experiments were carried out under defined conditions. Supplementation of culture media with AA, LA, CLA or SA yielded different results on cell proliferation. As shown in Fig. 2 and Table 1, the growth rate of cells incubated with AA during 5-day experiment was significantly higher than those of cells incubated with other fatty acids. Supplementation of 10 µM AA and LA in serum free DMEM increased cell growth of IEC-6 cells compared to those in 10 µM CLA and SA. The proliferation of cells supplemented with AA or LA were significantly higher than those of BSA. Cell growth analysis revealed the growth rate of cells incubated with AA or LA were significantly higher than that of cells incubated with CLA or SA. There was no significant difference in the growth rate of cells incubated with AA and LA. This is in agreement with Mollerup's study which revealed that low concentration of either LA or AA stimulated cell proliferation of the 21HKE cell, where as the saturated fatty acids, palmitic and stearic acid did not stimulate the proliferation of the 21 HKE cells (32). LA and AA also stimulated cell proliferation of murine colon adenocarcinomas (7). Studies have shown that the growth of human prostate (pc-3) and breast (HDA-MB 231) cancer cell lines were stimulated by low concentration of n-6 PUFA. Low concentration of n-3 PUFAs, EPA and DHA were growth inhibitory (34,35).

Increased rate of colon cell proliferation has been correlated with an increased tumorgenesis in the colon (18). Colon

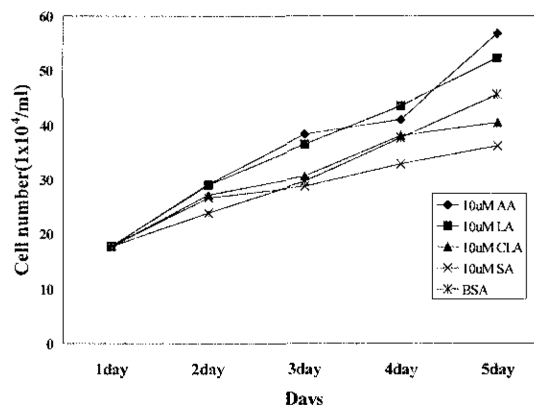

Fig. 2. Effects of supplementation of 10 µM arachidonic acid (AA), linoleic acid (LA), conjugated linoleic acid (CLA) and stearic acid (SA) on IEC-6 cell growth.

Table 1. Effects of free fatty acids on IEC-6 cell growth

	Days				
	1	2	3	4	5
AA	18.39±5.89	29.25± 7.55^{a}	38.31±15.16^{a}	40.42±13.34^{ab}	56.83±19.49^{a}
LA	18.39±5.89	29.03± 8.70^{a}	36.53±11.98^{a}	43.58±18.05^{a}	52.36±22.50^{ab}
CLA	18.39±5.89	27.22± 8.25^{ab}	30.72±11.05^{b}	38.11±15.43^{ab}	40.50±20.37^{cd}
SA	18.39±5.89	23.69±10.81^{b}	28.81±10.77^{b}	32.88±17.44^{b}	36.22±19.80^{d}
BSA	18.39±5.89	23.86±10.09^{b}	29.69± 9.75^{b}	37.67±18.85^{ab}	45.67±16.63^{bc}

Values are mean±standard deviation. n=9/group
Experiments were performed in triplicate and repeated 3 times. Statistical analysis was performed by ANOVA. Values with different letters within a column are significantly different from each other α=0.05 as determined by Duncan's multiple range test.
AA: 10 µM arachidonic acid, LA: 10 µM linoleic acid, CLA: 10 µM conjugated linoleic acid
SA: 10 µM stearic acid, BSA: 0.1% bovine serum albumin

mucosa cells near tumor sites were found to be more proliferative than those in normal tissue (19). Exogenous fatty acids may induce changes in the fatty acid profile of colonic mucosal cell membranes (17). At present, the mechanism through which an increased AA concentration in phospholipids leads to an increased cell proliferation is unknown. A major metabolic role of AA is its biotransformation to the series 2 prostaglandins. These molecules have very short half-lives and, therefore, are synthesized from AA which is released from cell membrane phospholipids by phopholipase. Evidence suggests that prostaglandin E2 is involved in certain types of carcinogenesis (36,37). Also, AA and its metabolites have been recently identified as a novel group of intracellular second messengers regulating activities of enzymes involved in signal transduction, such as protein kinase (38). It was found that AA activated mitogen-activated protein kinase (MAP kinase) in vascular smooth muscle cells, and protein kinase C appears to mediate AA induced activation of MAPkinase. AA can directly activate protein kinase C (39,40) and has been found to stimulate expression of early growth responsive genes such as c-fos and c-jun in vascular smooth muscle cells (15). Therefore, there are many possible roles in which AA might promote cell growth.

[^{3}H] thymidine incorporation

The effects of exogenous fatty acids (10 μM LA, AA, SA, CLA) on IEC-6 cell proliferation was assessed by [^{3}H] thymidine incorporation analysis. The [^{3}H] thymidine uptake experiment results were consistent with the cell growth analysis. As shown in Fig. 3 and Table 2, during the 4-day incubation, [^{3}H] thymidine uptake was increased in IEC-6 cells with AA and LA. As shown in Fig. 3 [^{3}H] thymidine uptake in cells incubated with 10 μM AA or LA were significantly higher than in cells supplemented with either CLA or SA. AA or LA cells were only slightly higher than that of cells cultured with serum free media (BSA). A synergistic effect of IGF-1 was observed. IGF-1 increased [^{3}H] thymidine uptake into IEC-6 cells under each treatment and this stimulation was higher in cells incubated with AA or LA compared with cells treated with CLA, SA, or BSA. Sauer and Dauchy (41) showed that LA was about three times more effective than the other n-6 fatty acids. In this study [^{3}H] thymidine uptake in cells incubated with 10 μM AA is more effective than that of LA. Murine masto cytoma cells cultured in serum free media with AA grew at 50~70% of the rate of cells in 10% FBS, but without AA cells did not grow (13).

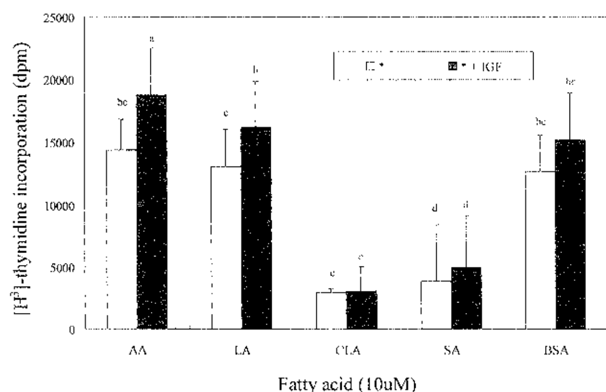

Fig. 3. [^{3}H]-thymidine incorporation in IEC-6 cell incubated with 10 μM arachidonic acid, linoleic acid, conjugated linoleic acid and stearic acid with or without IGF-1 stimulation. The open bar represents thymidine uptake in cells stimulated without IGF-1, the solid bar represents the level of thymidine uptake with IGF-1 stimulation. Values are mean ± standard deviation. Values with different letters are significantly different from each other at α=0.05 as determined by Duncan's multiple range test. AA: arachidonic acid, LA: linoleic acid, CLA: conjugated linoleic acid, SA: stearic acid, BSA: 0.1% bovine serum albumin, IGF: 3×10^{-8} M insulin like growth factor.

Tyrosine phospholylation

Tyrosine kinase activity of the receptor is a prerequisite for IGF- mediated signal transduction. To study the mechanism involved, the effect of AA, LA and CLA on tyrosin kinase activity was investigated. Cells were then exposed to IGF for 3 min. Cellular extracts were subjected to immunoblotting and probed with a phosphotyrosine specific antibody.

As shown in Fig. 4, the basal level of tyrosine phosphorylation on the IGF-R was highest in cells cultured in 10 μM AA, lowest in 20 μM CLA. IGF-1 significantly increased tyrosine phosphorylation on the IGF receptor. AA, at 10 μM concentration or 20 μM concentration, stimulated tyrosine kinase activity in the IEC-6 cell. AA stimulated IGF-induced tyrosine phosphorylation dose independently. The stimulatory effect of AA was even more profound in the presence of IGF. LA was less effective in IGF-receptor autophosphorylation than AA. 10 μM LA with IGF yielded more reduction in IGF-receptor autophosphorylation than 20 μM LA with IGF. CLA and CLA with IGF-1 inhibited protein tyrosine phosphorylation in IEC-6 cells at both concentrations. This is in agreement with the work of Bandyopadhyay and

Table 2. [^{3}H]-thymidine incorporation of IEC-6 cell incubated with 10 μM of arachidonic acid, linoleic acid, conjugated linoleic acid and stearic acid

Fatty acid	[^{3}H]-thymidine incorporation DPM/plate
AA	14374.09 ± 2474.28^{bc}
AA + IGF	18750.78 ± 3720.24^{a}
LA	13052.09 ± 3013.14^{c}
LA + IGF	16242.96 ± 3906.03^{b}
CLA	2987.98 ± 279.44^{c}
CLA + IGF	3085.52 ± 1965.54^{c}
SA	3861.31 ± 4514.94^{d}
SA + IGF	4959.88 ± 4187.60^{d}
BSA	12689.36 ± 2888.71^{bc}
BSA + IGF	15174.78 ± 3740.05^{bc}

Experiments were performed in triplicate and repeated 3 times.
Statistical analysis was performed by ANOVA.
Values are mean ± standard deviation
Values with different letters within a column are significantly different from each other at α=0.05 as determined by Duncan's multiple range test.
AA: 10 μM arachidonic acid, LA: 10 μM linoleic acid,
CLA: 10 μM conjugated linoleic acid, SA: 10 μM stearic acid,
BSA: 0.1% bovine serum albumin
IGF: 3×10^{-8} M insulin like growth factor

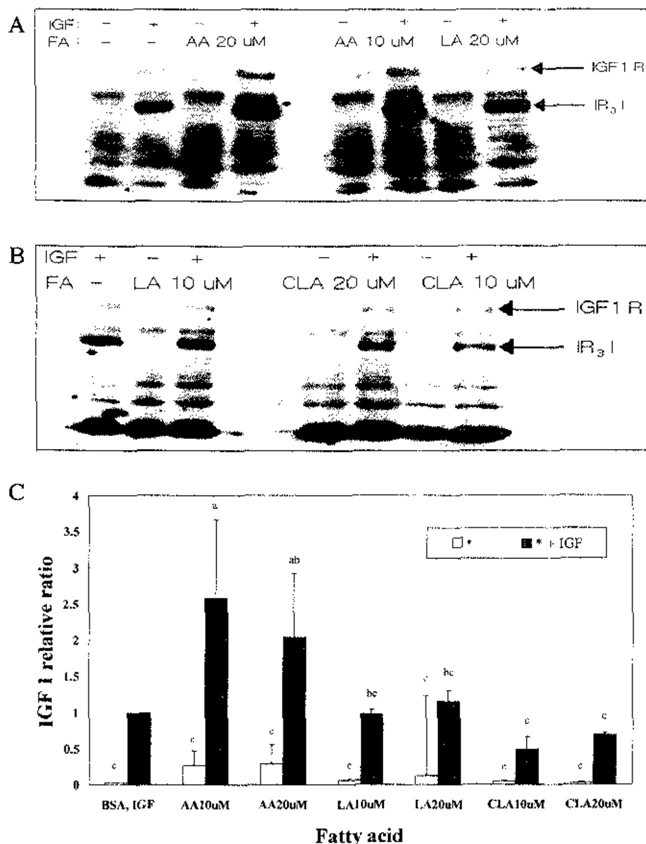

Fig. 4. Effect of arachidonic acid, linoleic acid and conjugated linoleic acid on IGF receptor autophosphorylation *in vitro*. A,B: The cells were left either unstimulatd with IGF-deprivation (-) or stimulated with 3×10^{-8} M IGF for 3 min (+). C: Relative levels of IGF receptor autophosphorylation were quantitated using a Betascope (Betgen). The data are in ratio of IGF. Values with different letters are significantly different from each other at α=0.05 as determined by Duncan's multiple range test. AA: arachidonic acid, LA: linoleic acid, CLA: conjugated linoleic acid, BSA: 0.1% bovine serum albumin, IGF: 3×10^{-8} M insulin like growth factor.

Hwang (42), who demonstrated that PUFAs n-6, n-7 and n-9 classes modulated EGF mediated signal transduction. However, studies on the action of n-3 PUFAs on signal transduction are limited. Tomaska and Resnick (43) showed exposure of platelet-derived growth factor receptors to increasing concentrations of AA resulted in the progressive reduction in its autophosphorylation. Though this study experimented at only two levels, 10 μM and 20 μM, 20 μM AA with IGF yielded more reduction in IGF-receptor autophosphorylation than 10 μM AA with IGF. This indicates that the arachidonate signal is transduced, in part, through tyrosine kinase. As in several studies, AA or LA modulates the activity of enzymes and proteins such as protein kinase A (44), protein kinase C (45) and GTP-binding protein (46). LA was less effective than AA. This study also showed the same results.

In summary, this study suggests that IEC-6 cell proliferation was influenced differently by exogenous free fatty acids, in which AA or LA stimulated cell proliferation and CLA inhibited cell proliferation. There is a possible correlation between cell proliferation and the IGF receptor tyrosine kinase activity driven by AA. Further studies are needed to determine other specific mechanisms involved.

ACKNOWLEDGEMENTS

This paper was accomplished with 1997 Reseach Fund provided by Korea Research Foundation, Support for Faculty Research Abroad.

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(Received August 5, 1999)