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Mechanism for the Antitumor and Anticachectic Effects of n-3 Fatty Acids[1]

Leonard A. Sauer,[2] Robert T. Dauchy, and David E. Blask

Bassett Research Institute, Cooperstown, New York 13326

ABSTRACT

Dietary intake of the n-6 fatty acid (FA) linoleic acid (LA) has a strong growth-promoting effect on many rodent tumors and human tumor xenografts grown in immunodeficient rodents. n-3 FAs such as α-linolenic and eicosapentaenoic acids (EPAs), which differ from LA and arachidonic acid, respectively, by only a single double bond in the n-3 position, are recognized cancer chemopreventive and anticachectic agents. Understanding how this seemingly small structural difference leads to such remarkable functional differences has been a challenge. In a previous study, we showed that LA uptake, [^{3}H]thymidine incorporation into DNA, and total DNA content were decreased in tissue-isolated hepatoma 7288CTC perfused *in situ* with arterial blood containing α-linolenic acid, EPA, or docosahexaenoic acids. The K_i for the inhibition of LA uptake and [^{3}H]thymidine incorporation by α-linolenic acid was 0.18 and 0.25 mM, respectively. Here we show that the addition of α-linolenic acid or EPA to arterial blood inhibits tumor FA uptake, including LA, and the subsequent conversion of LA to the mitogen 13-hydroxyoctadecadienoic acid (13-HODE) *in vivo* and during perfusion *in situ*. [^{3}H]Thymidine incorporation during perfusion *in situ* was also inhibited. Addition of 13-HODE to the arterial blood reversed the inhibition of [^{3}H]thymidine incorporation but had no effect on FA uptake. These two n-3 FAs also inhibited FA transport in inguinal fat pads *in vivo* and during perfusion *in situ* in fed (FA uptake) and fasted (FA release) rats. The effects of EPA and α-linolenic acid on transport of saturated, monounsaturated, and n-6 polyunsaturated FAs in hepatoma 7288CTC and inguinal fat pads during perfusion *in situ* were reversed by the addition of forskolin (1 μM), pertussis toxin (0.5 μg/ml), or 8-bromo-cyclic AMP (10 μM) to the arterial blood. We conclude that the antitumor and anticachectic effects of n-3 FAs on hepatoma 7288CTC and inguinal fat pads *in vivo* result from an inhibition of FA transport. These inhibitions are mediated by a putative n-3 FA receptor via a G_i protein-coupled signal transduction pathway that decreases intracellular cyclic AMP. A specific decrease in LA uptake and its conversion to the mitogen 13-HODE causes the tumor growth inhibition.

INTRODUCTION

Nutritional, biochemical, and other experimental studies performed over the last 60–70 years have provided convincing evidence that dietary fat plays an important role in tumorigenesis and the growth of established tumors in rodents *in vivo* (1–4). These experiments also revealed several interesting biochemical puzzles that have not yet been resolved. For example, consumption of LA[3] (C18:2n6; Ref. 4)-enriched diets increased tumorigenesis (5, 6) and the growth of transplanted tumors in rodents (7, 8) and the growth of human breast cancer xenografts in immunodeficient rodents (9, 10). In contrast, consumption of diets containing n-3 FAs, including α-linolenic acid (C18:3n3), EPA (C20:5n3), or docosahexaenoic acid (C22:6n3), inhibited tumorigenesis (11, 12) and the growth of rodent tumors (13, 14) and human breast cancer xenografts (15, 16). α-Linolenic acid differs from LA by the presence of one double bond at the n-3 position. EPA, an n-3 FA that is a potent tumor growth inhibitor, differs from arachidonic acid (C20:4n6) by the n-3 double bond. Dietary arachidonic acid does not stimulate the growth of rodent tumors *in vivo* (7, 8). Understanding how these seemingly small structural differences could lead to such remarkable functional differences has been a challenge.

Research in this laboratory has examined the effects of n-6 and n-3 FAs on the growth of tissue-isolated implants of hepatoma 7288CTC, a transplantable rat tumor. Hepatoma 7288CTC showed a direct relationship between the rates of tumor LA uptake, formation of 13-HODE from LA, and tumor growth *in vivo* (8). During perfusion *in situ*, the incorporation of [^{3}H]thymidine into tumor DNA was directly proportional to plasma LA concentration and LA uptake (17) and 13-HODE formation (18). Tumor growth *in vivo* and [^{3}H]thymidine incorporation during perfusion *in situ* were inhibited by a lipoxygenase inhibitor (18), which blocked 13-HODE formation, and by melatonin, which blocked tumor FA uptake and 13-HODE formation (19). The effects of the lipoxygenase inhibitor and melatonin were reversed by the addition of 13-HODE to the arterial blood during perfusion *in situ*. We concluded from these experiments that 13-HODE is the mitogenic agent responsible for LA-dependent growth in hepatoma 7288CTC and suggested that it may have a similar role in the growth of other rodent tumors and in human breast cancer xenografts grown in immunodeficient rodents.

Elucidation of these roles of LA uptake and 13-HODE formation in hepatoma 7288CTC suggested a new approach to the question of the interrelationships between n-6 and n-3 FAs and tumor growth. In 1992, we observed that the addition of α-linolenic, EPA, or docosahexaenoic acid to the arterial blood during perfusion of hepatoma 7288CTC *in situ* inhibited tumor LA uptake and the rate of incorporation of [^{3}H]thymidine into tumor DNA (17). Uptake of all other saturated, monounsaturated, and n-6 PUFAs was also inhibited. K_i values for α-linolenic acid were 0.18 mM for LA uptake and 0.25 mM for [^{3}H]thymidine incorporation. There was only a small uptake of the n-3 FAs by hepatoma 7288CTC. We proposed that plasma n-3 FAs inhibited tumor growth *in vivo* and [^{3}H]thymidine incorporation during perfusion *in situ* by competitively inhibiting LA uptake. However, at that time, evidence for the presence of specific cellular FATPs for either n-6 or n-3 FAs in either normal or tumor cells was controversial (20, 21), and, other than the kinetic data, we had no additional experimental support for this proposal.

In this report, we examine the role of α-linolenic acid and EPA on uptake of plasma FAs and 13-HODE formation in hepatoma 7288CTC *in vivo* and during perfusion *in situ*. The results provide strong evidence that n-3 FAs inhibit tumor growth by blocking tumor FA uptake and 13-HODE formation. Similar effects of EPA and α-linolenic acid were observed on FA transport in inguinal fat pads in fasted tumor-bearing rats *in vivo* and in fed or fasted normal rats during perfusion *in situ*. The evidence presented suggests that n-3 FAs inhibit uptake of other plasma FAs in tumor and adipose tissue via a

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[2] To whom requests for reprints should be addressed, at Bassett Research Institute, One Atwell Road, Cooperstown, NY 13326. Phone/Fax: (406) 777-4360; E-mail: lensauer@juno.com.

[3] The abbreviations used are: LA, linoleic acid; FA, fatty acid; PUFA, polyunsaturated FA; 13-HODE, 13-hydroxyoctadecadienoic acid; EPA, eicosapentaenoic acid; A-V, arteriovenous difference; FFA, free fatty acid; TAG, triacylglycerol; PL, phospholipid; CE, cholesterol ester; PTX, pertussis toxin; cAMP, cyclic AMP; 8-Br-cAMP, 8-bromo-cyclic AMP; FATP, fatty acid transport protein; GC, gas chromatography.

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putative n-3 FA receptor-mediated, G_i protein-coupled signal transduction pathway that reduces the intracellular cAMP concentration.

MATERIALS AND METHODS

Animals, Diets, and Tumor Implantation. Male Buffalo rats and male Sprague Dawley rats were purchased from Charles River Laboratories (Wilmington, MA) and Harlan Sprague Dawley (Indianapolis, IN), respectively. Both strains were specific pathogen free and were maintained at 23°C and 45–50% humidity in microisolator units (Thoren Caging Systems, Hazelton, PA) in a facility approved by the American Association for Accreditation of Laboratory Animal Care. Lighting was diurnal [12-h light/12-h dark cycle (lights were on from 6 a.m. to 6 p.m.; 300 lux)]; there was no light leak during the dark period. Animals were given free access to water and chow (Prolab 1000 Formula; Agway, Inc., Syracuse, NY). Analysis of several batches of this diet indicated that the FA content was 4.2 g FAs/100 g, with palmitic acid (21%), stearic acid (11%), oleic acid (34%), and LA (28%) as the major FAs. In some experiments, the rats were fasted for 48 h.

Hepatoma 7288CTCs were implanted in male Buffalo rats as tissue-isolated tumors as described previously (17–19, 22–26). Briefly, a 3-mm^3 tumor was implanted on the tip of a vascular stalk formed from the superficial epigastric artery and vein. The implant and vascular stalk were enclosed within a parafilm envelope and placed in the inguinal fossa, and the skin incision was closed. Vascularization of the implant was limited to new vessel connections with the epigastric artery and vein, and tumor growth was s.c. The latent period from implantation to first evidence of tumor growth was recorded, and subsequent growth was estimated every 2–3 days from measurements made through the skin (23). These data were converted to weights, and the growth rates (grams/day) were calculated by linear regression (23).

A-V Measurements across Hepatoma 7288CTC *in Vivo* and during Perfusion *in Situ*. Experiments were performed when the estimated tumor weights were 4–6 grams. A-V measurements *in vivo* were performed between 8 and 10:30 a.m. after a normal nocturnal feeding period (except for the fasted rats). Procedures for anesthesia, heparinization, surgical preparation, and maintenance of body temperature of the host rat and collection of arterial and tumor venous blood samples across the tumor were performed as described previously (8, 19, 22–26). Blood flow from the tumor vein was 0.11–0.13 ml/min, and blood was collected passively. Anesthetized host rats were breathing air unassisted.

Detailed descriptions of the surgical and technical procedures for perfusion of tissue-isolated hepatoma 7288CTC *in situ* were described in previous reports (17–19, 24–26). Donor blood for perfusion (~50 ml) was collected between 8 and 10 a.m. from either fed or 48-h-fasted Sprague Dawley rats weighing 250–300 grams. Rats were anesthetized with sodium pentobarbital (Abbott Laboratories, North Chicago, IL; 25 mg/kg body weight; i.p.), and anticoagulated by i.v. infusion of sodium heparin (Elkins-Sinn, Inc., Cherry Hill, NJ; 50 units/100 g body weight). Arterial blood was collected from a carotid catheter, filtered through cheesecloth, and stored under mineral oil in a stirred plastic reservoir chilled in ice. This whole-blood perfusate was pumped from the reservoir through a 37°C water bath and an artificial lung using a peristaltic pump (Model 1215; Harvard Apparatus, Natick, NA). The pH, pO_2, and pCO_2 in samples collected from the arterial catheter were monitored using a blood gas analyzer (Model 995; AVL, Graz, Austria) and maintained at 7.4 and 40 and 100 mm Hg, respectively. The vascular connections (epigastric artery and vein) between tumor and host were severed after attachment of the tumor to the arterial catheter. The host was sacrificed, but the hindquarters containing the tumor were monitored and maintained at 37°C throughout the perfusion. A diagram depicting the perfusion system is shown in Fig. 1 in Ref. 26. Calculations of the rates of blood flow, nutrient uptake, and 13-HODE release were based on actual tumor weights measured at the end of the experiment.

The purpose of these experiments was to ensure that steady-state rates of tumor FA uptake and metabolism and DNA synthesis were established and to simulate the *in vivo* conditions of hyperlipemia that are associated with rapid tumor growth and cachexia (27). Previous experiments (17–19, 25) showed that 15–20 min were required for steady states to be established in tumors perfused *in situ* and that increased plasma FA concentrations increased the rates of tumor FA uptake (17, 24). Perfusions were usually 150 min in duration and were preceded by a 30-min perfusion period (no sample collection) to establish a steady state. Arterial and tumor venous blood samples were collected into chilled tubes at 30-min intervals and stored in ice for analysis of FA, glycerol, and 13-HODE. Arterial blood samples were collected from the catheter just before entry into the tumor. Depending on the experiment, the donor blood was supplemented with either EPA or α-linolenic acid (plasma concentration, 0.3–0.9 mM) with or without PTX (0.5 μg/ml), forskolin (1 μM), 8-Br-cAMP (10 μM), or 13-HODE (0.4 ± 0.03 μM). The incorporation of [^{3}H]thymidine into tumor DNA was performed by injecting 20 μl of a solution containing 2 μCi [methyl-[^{3}H]]thymidine/gram of estimated tumor weight into the arterial catheter 20 min before the end of the perfusion (17–19, 25). The [^{3}H]thymidine made one pass through the tumor and was washed out during the remaining 20 min of perfusion. Radioactivity incorporated into tumor DNA was measured by liquid scintillation using internal standards and is reported as dpm/μg tumor DNA. DNA was measured fluorometrically in 20% homogenates using Hoechst dye 33258 (Hoefer Scientific Instruments, San Francisco, CA), and the procedure was as described previously (28).

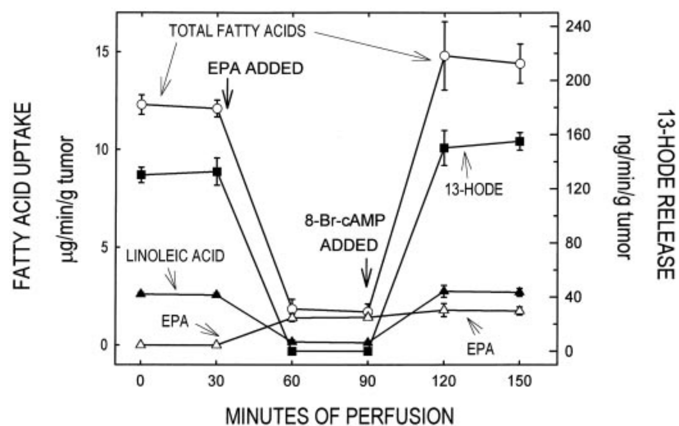

Fig. 1. Effects of EPA and 8-Br-cAMP on FA uptake and 13-HODE release in hepatoma 7288CTC perfused *in situ*. Arterial and tumor venous blood samples were collected at zero time and at 30-min intervals. EPA (0.68 ± 0.02 mM in arterial blood plasma) was added at 36 min, and 10 μM 8-Br-cAMP was added at 96 min. Each point represents the mean ± SD for three perfusions. Tumor venous blood flow = 0.11–0.13 ml/min. Host and blood donor rats were fasted for 48 h to increase blood lipid levels. Mean tumor weight was 5.8 ± 0.4 g. The absence of an error bar indicates that the SD was within the point symbol.

In some experiments, the kinetic effects of n-3 FAs and other agents on tumor FA transport and 13-HODE formation were measured. The 150-min perfusion period was divided into two or three periods, as follows: (*a*) 66 min after the start of perfusion with untreated donor blood (either fed or fasted rats), n-3 FA was added to the reservoir to give a final plasma concentration of 0.3–0.9 mM, and the perfusion was continued until 150 min; and (*b*) after a control perfusion for 36 min, the n-3 FA was added, and the perfusion was continued; at 96 min, PTX (0.5 μg/ml), forskolin (1 μM), or 8-Br-cAMP (10 μM) was added to the blood containing n-3 FA, and the perfusion was continued until 150 min. Arterial and tumor venous blood samples were collected every 30 min. At completion of the perfusion, the tumor was removed, weighed, and frozen at −20°C. Whole blood samples were centrifuged for 10 min at 10,000 × *g* (4°C), and plasma was collected and frozen at −20°C.

A-V Measurements across Inguinal Fat Pads *in Vivo* and during Perfusion *in Situ*. The procedures described above for A-V blood collections across tissue-isolated tumors were modified for A-V measurements across the inguinal fat pad. The *in vivo* measurements were made across a tumor and inguinal fat pad simultaneously in the same tumor-bearing rat. The host rat was anesthetized and heparinized, and the tumor (on the animal's left side) was prepared for A-V measurement as described above. The right inguinal fat pad was prepared as follows: the epigastric vessels supplying the caudal pole of the fat pad were exposed by a 3-cm incision in the right inguinal fossa. A butterfly infusion catheter (number 4573; Abbot Hospital Products, North Chicago, IL) was inserted into the vein, draining the fat pad. Venous blood was allowed to flow passively (the venous blood flow rates from the fat pads were about 80 μl/min). Blood samples (~0.5 ml) were collected simultaneously from the fat

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pad and tumor veins. An arterial blood sample was collected from the catheter in the carotid artery midway through the venous sample collections. EPA (sodium salt in saline) estimated to give a plasma concentration of 0.3–0.5 mM in a 250- to 300-gram rat with a whole blood volume of ~10 ml was infused into a catheter in the jugular vein. A second set of blood samples was collected 1–2 min later. At the end of the experiment, the fat pad and tumor were excised, weighed, and frozen at −20°C.

The procedure used for perfusion of the left inguinal fat pad *in situ* in non-tumor-bearing rats was as follows. The caudal epigastric artery and vein supplying the fat pad were exposed, and the vein was cannulated as described above. The catheter carrying the warmed, oxygenated donor blood from the reservoir was inserted into the femoral-epigastric arterial trunk leading to the fat pad. The pump settings were adjusted to provide a flow from the venous catheter of about 80 μl/min. When a uniform venous flow rate was established, the femoral artery and vein distal to the fat pad were ligated, and the host was exsanguinated through the carotid catheter. Death of the host did not affect the venous flow from the fat pad. Arterial and venous blood samples were collected at 30-min intervals, and treatments with n-3 FAs, PTX, forskolin, and 8-Br-cAMP were as described above. A-V measurements *in vivo* and during perfusion *in situ* were performed between 7:30 a.m. and 1 p.m. The surgical and experimental procedures were approved by the Institutional Animal Care and Use Committee.

Lipid Extraction and Analysis. Total plasma lipids were extracted from 0.2 ml of arterial or venous plasma after the addition of internal standards (8, 17–19, 24, 25). Duplicate extracts for FA analysis were saponified, methylated, and assayed by GC. In some experiments, the arterial and venous plasma samples (containing four added internal standards) were extracted; separated by TLC into FFA, TAG, PL, and CE lipid fractions; eluted from the plates; saponified; and methylated; and the FA contents were analyzed by GC (24). 13-HODE was measured by high-performance liquid chromatography as described previously (8, 18, 19). A-V measurements were converted to rates of FA uptake or release and expressed as micrograms or micromoles/minute/gram fat pad or tumor. 13-HODE release was expressed as nanograms/minute/gram tumor. Unless otherwise indicated, total FA represents the sum of the seven major plasma FAs [myristic acid, palmitic acid, palmitoleic acid, stearic acid, oleic acid, LA, and arachidonic acid (and EPA, when present)] in rat blood plasma. Plasma concentrations for total FA, LA, EPA, and α-linolenic acid are given as micromolar ± 1 SD, unless otherwise indicated.

Glycerol Analysis. Glycerol concentrations were measured spectrophotometrically using an Ultraspec 4000 UV/visible spectrophotometer equipped with a Compaq Deskpro 2000 computer (Pharmacia Biotech, Ltd., Piscataway, NJ). Perchloric acid extracts of plasma (22, 26) derived from the arterial and venous blood collections were analyzed using a kit purchased from Sigma/Aldrich (St. Louis, MO). Glycerol release from the fat pads is reported as micrograms/minute/gram fat pad.

Statistical Analysis. Results are expressed as mean ± SD and were compared using one-way ANOVA followed by a Student-Newmann-Keuls multiple-comparison test (29). Differences among the groups were considered statistically significant at $P < 0.05$.

RESULTS

Effects of EPA on FA Transport in Hepatoma 7288CTC and Inguinal Fat Pads *in Vivo*. Simultaneous A-V measurements across hepatoma 7288CTCs and inguinal fat pads *in vivo* in fasted tumor-bearing rats showed that plasma FAs, including LA, were removed from the arterial blood by the tumors and released into the venous blood by the inguinal fat pads (Table 1). Negative values (more FAs in venous blood than in arterial blood) represent FA release; positive values represent FA uptake. The rate of FA uptake by the tumor was nearly double the rate of FA release from the right inguinal fat pad. In the fasted tumor-bearing rat, relative to the fed rat, the plasma concentration of LA (8) and the rates of tumor 13-HODE formation and growth were increased (8, 18). After collection of the first set of arterial blood samples and two venous blood samples, EPA (sodium salt, in saline) was injected into the host via the jugular vein catheter; the second set of blood samples was collected 1 min later. Total collection time was about 10 min. The presence of EPA in the arterial blood inhibited the uptake of plasma FAs in the tumor, except for that of EPA itself, and completely inhibited FA release by the inguinal fat pad. The rate of total FA uptake by hepatoma 7288CTCs after EPA injection was accounted for by the uptake of EPA; the values for total FA and EPA uptakes were not different ($P > 0.05$). As a result of the block in LA uptake, tumor release of 13-HODE was inhibited. 13-HODE is undetectable in rat arterial blood plasma (8). Fat pads did not release 13-HODE into the venous blood.

Changes in FA Uptake in Hepatoma 7288CTC Induced by EPA and 8-Br-cAMP during Perfusion *in Situ*. The kinetics of the changes in total plasma FA and LA uptakes and 13-HODE release by hepatoma 7288CTC following consecutive additions of EPA and 8-Br-cAMP to the arterial blood are shown in Fig. 1. Steady-state rates of total FA and LA uptake and 13-HODE release were evident during the first 30 min of perfusion. The endogenous EPA concentration in the donor arterial blood was ≤40 μM, and uptake was too small to be measured accurately. The addition of EPA to the arterial blood at 36 min promoted an EPA uptake of 1.41 ± 0.15 μg/min/g tumor and inhibited the uptake of total plasma FAs, including LA. In the absence of LA uptake, release of 13-HODE stopped. As shown in Table 1, the rate of FA uptake by hepatoma 7288CTC following the addition of EPA was accounted for by the uptake of EPA itself. The addition of 8-Br-cAMP to the perfusate returned the rates of tumor total FA and LA uptakes and 13-HODE release to pre-EPA rates. The stimulation by 8-Br-cAMP of the uptake of plasma-saturated, monounsaturated, and n-6 PUFAs occurred, despite the presence of EPA in the perfusate. In separate experiments (data not shown), we found that α-linolenic acid (0.21 mM plasma concentration) was as effective as EPA in blocking FA uptake and 13-HODE release; the action of α-linolenic acid was also reversed by 8-Br-cAMP. PTX and forskolin were as effective as 8-Br-cAMP in reversing the inhibition of FA transport by either EPA or α-linolenic acid.

Steady-State Rates of FA Uptake, 13-HODE Release, and [^{3}H]Thymidine Incorporation in Hepatoma 7288CTC Perfused *in Situ*: The Effect of EPA and EPA plus Forskolin, 8-Br-cAMP, PTX, or 13-HODE. In these experiments, 18 tumor-bearing rats were divided into a control group and five treatment groups (three rats/group). The rats in the six groups and the blood donor rats were fasted for 48 h before the

Table 1 *Effect of EPA on uptake of plasma total FAs and LA and release of 13-HODE by hepatoma 7288CTC and release of total FAs by the inguinal fat pad in vivo in fasted male Buffalo rats*[a]

	Hepatoma 7288CTC				Inguinal fat pad	
	Total FA uptake[b] (μg/min/g)	LA uptake (μg/min/g)	13-HODE release (ng/min/g)	Blood flow (μl/min)	Total FA uptake (μg/min/g)	Blood flow (μl/min)
Before EPA	10.4 ± 1.5	2.5 ± 0.4	156 ± 10	129 ± 4	−6.9 ± 1.2	82 ± 2
After EPA[c]	0.6 ± 1.0[d]	0.01 ± 0.5	ND[e]	124 ± 3	0.2 ± 0.5	80 ± 2

[a] A-V measurements were collected simultaneously across the tumor and inguinal fat pad in a tumor-bearing rat. The values are the means ± SD from A-V measurements across three tumor-bearing rats.
[b] Total FAs represent the sum of myristic acid, palmitic acid, palmitooleic acid, stearic acid, oleic acid, LA, and arachidonic acid and EPA, when present.
[c] The arterial blood plasma concentrations for EPA in the three experiments were 0.27, 0.56, and 0.33 mM, respectively.
[d] This value includes the mean rate of uptake of EPA, which was 0.52 ± 0.07 μg/min/g tumor.
[e] ND, not detected.

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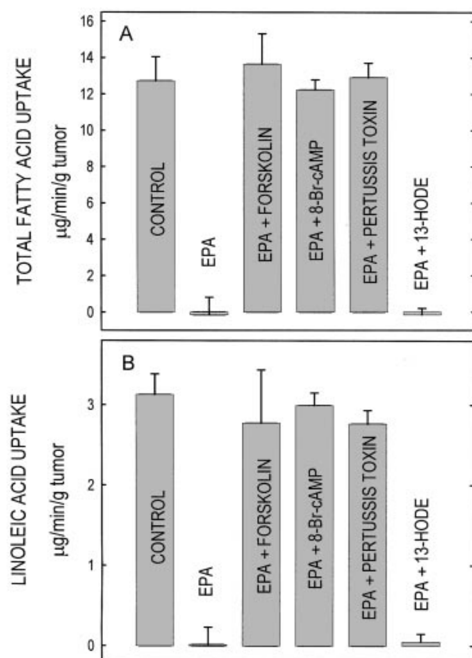

Fig. 2. Effect of EPA, EPA + forskolin, EPA + 8-Br-cAMP, EPA + PTX, or EPA + 13-HODE on uptake of total FAs (*A*) and LA (*B*) in hepatoma 7288CTC perfused *in situ*. Perfusions were for 150 min in the presence of the components indicated. There were no additions to the arterial blood in the control perfusions. Host and blood donor rats were fasted for 48 h before the start of the experiments. Blood samples were collected at 30-min intervals, and column values represent the mean ± SD for 18 samples. The concentrations of forskolin, 8-Br-cAMP, and PTX were 1 μM, 10 μM, and 0.5 μg/ml plasma, respectively. The arterial blood plasma:EPA concentration was 0.45 ± 0.02 mM for the 15 perfusions. 13-HODE concentration for the three perfusions was 0.40 ± 0.02 μM. Mean tumor weight was 6.6 ± 0.3 g (n = 18).

start of the experiments. Mean plasma concentrations for the major FAs in fasted donor arterial blood (six batches, 50 ml each) were as follows: (*a*) myristic acid, 20 ± 5 μM; (*b*) palmitic acid, 1.28 ± 0.06 mM; (*c*) palmitoleic acid, 0.13 ± 0.03 mM; (*d*) stearic acid, 0.83 ± 0.04 mM; (*e*) oleic acid, 1.06 ± 0.14 mM; (*f*) LA, 1.59 ± 0.14 mM; (*g*) arachidonic acid, 1.93 ± 0.17 mM; and (*h*) EPA, 30 ± 10 μM. A-V measurements across tumors in the control group represented the baseline steady-state rates for FA uptake, 13-HODE release, and [^{3}H]thymidine incorporation. Arterial blood perfusates for the five treatment groups also contained added EPA, EPA + 1 μM forskolin, EPA + 10 μM 8-Br-cAMP, EPA + 0.5 μg/ml PTX, or EPA + 0.40 ± 0.02 μM 13-HODE, respectively. The final mean plasma EPA concentration in the arterial blood used for perfusion of the five treatment groups was 0.52 ± 0.09 mM. The 13-HODE concentration added to the arterial blood was calculated to equal or exceed the concentrations observed in tumor venous blood plasma in fasted rats.

Comparisons of the steady-state rates of total FA and LA uptakes by tumors in the control and treated groups are shown in Fig. 2, *A* and *B*, respectively. The addition of EPA caused a complete inhibition of total FA and LA uptake. These inhibitions attributable to EPA were reversed in the presence of either forskolin, 8-Br-cAMP, or PTX. The addition of EPA + 13-HODE did not reverse the inhibited uptake of FA. EPA uptake in the control tumor group was too low to be measured accurately. However, EPA uptake was observed in the EPA treatment groups as follows: (*a*) EPA, 0.40 ± 0.09 μg/min/g tumor; (*b*) EPA + forskolin, 1.02 ± 0.07 μg/min/g tumor; (*c*) EPA + 8-Br-cAMP, 0.98 ± 0.05 μg/min/g tumor; (*d*) EPA + PTX, 1.70 ± 0.13 μg/min/g tumor; and (*e*) EPA + 13-HODE, 0.42 ± 0.03 μg/min/g tumor. Each value represents 16 measurements for each treatment group. Rates of EPA uptake were low compared with the uptake of either total FA or LA (Fig. 2). However, analysis (ANOVA) of these rates, normalized to a constant rate of EPA supply to the tumor, indicated that forskolin, 8-Br-cAMP, and PTX significantly increased EPA uptake ($P < 0.05$) relative to that in the EPA-treated and EPA + 13-HODE-treated groups.

EPA inhibited the rate of tumor-13-HODE release observed in the control group (Fig. 3*A*). The addition of either forskolin, 8-Br-cAMP, or PTX, which reversed the EPA inhibition of LA uptake, restored tumor 13-HODE release to control rates. Thus, EPA did not directly affect the enzymatic generation of 13-HODE from LA. Also, despite the presence of EPA, hepatoma 7288CTC removed significant amounts of 13-HODE from the arterial blood in the EPA + 13-HODE-treated group. Fig. 3*B* shows the rate of [^{3}H]thymidine incorporation that occurred in control tumors. LA-dependent synthesis and release of 13-HODE supported [^{3}H]thymidine incorporation (18). The presence of EPA abolished 13-HODE release (Fig. 3*A*) and [^{3}H]thymidine incorporation into hepatoma 7288CTC (Fig. 3*B*). This inhibition was reversed by the presence of forskolin, 8-Br-cAMP, or PTX. The addition of 13-HODE restored [^{3}H]thymidine incorporation, although EPA was present, and LA uptake was inhibited (Fig. 2*B*).

Effects of EPA on FA Release and Uptake by Inguinal Fat Pads Perfused *in Situ*. Inguinal fat pads in fasted rats perfused *in situ* with arterial blood from fasted donor rats released FAs into the venous blood. In contrast, inguinal fat pads in fed rats removed FAs from the arterial blood of fed donor rats. To determine the plasma lipid classes involved in these A-V differences, we extracted plasma lipids from arterial and venous blood samples; separated the lipids into TAG, PL, CE, and FFA fractions by TLC; and analyzed the constituent FAs by GC. The A-V differences measured across the fat pads were accounted for by changes in the plasma FFA content. FA contents of CEs, TAGs, or PLs were not different in arterial or venous plasma (data not shown). The mean rates (± SD; n = 16) of FFA release (−1.34 ± 0.08 μg/min/g fat pad) and uptake (3.66 ± 0.12 μg/min/g fat pad) were constant during the 150-min perfusions.

Fig. 4*A* shows that release of FFAs and glycerol from inguinal fat pads in fasted rats became inhibited when EPA was added 66 min after the start of the perfusion. The inhibited rates of FFA and glycerol release were completely restored to pre-EPA rates after the addition of 8-Br-cAMP to the arterial blood (Fig. 4*B*). Although trace amounts of EPA may have entered the fat pads, there was no measurable EPA

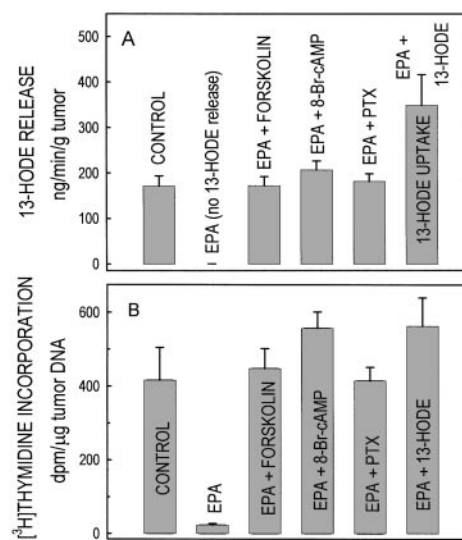

Fig. 3. Effect of EPA, EPA + forskolin, EPA + 8-Br-cAMP, EPA + PTX, or EPA + 13-HODE on 13-HODE release (*A*) and [^{3}H]thymidine incorporation (*B*) in hepatoma 7288CTC perfused *in situ*. Perfusion conditions, component concentrations, and tumor weights were as described in the legend to Fig. 2. The column for 13-HODE (*A*) represents an uptake of the added 13-HODE.

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uptake. In Fig. 4, *A* and *B*, the molar ratio of FA release:glycerol release was 0.5–0.6, suggesting that FA reesterification occurred in the inguinal fat pads. Although an internal standard (pentadecanoic acid) was added to correct for FFA losses during extraction and chromatography, and antioxidants were added to the TLC solvent system, PUFAs are subject to loss by autooxidation during TLC. Glycerol may also have been produced in excess.

Fig. 5 shows the rates of total FA uptake and glycerol release in inguinal fat pads in fed rats during perfusion *in situ*. Glycerol was released during periods of net FA uptake. The addition of EPA to the arterial blood 36 min after start of the perfusion inhibited uptake of FAs from the arterial blood and glycerol release (Fig. 5*A*). Despite the inhibition of plasma saturated, monounsaturated, and n-6 PUFA uptake, low but measurable rates of EPA uptake persisted. The addition of PTX restored uptake of the saturated, monounsaturated, and n-6 PUFAs but did not affect EPA uptake significantly (Fig. 5*B*). PTX, 8-Br-cAMP, and forskolin (data not shown) were equally effective in reversing the inhibition of FFA release or uptake caused by EPA or α-linolenic acid.

DISCUSSION

The consecutive reactions that control LA-dependent growth in hepatoma 7288CTC *in vivo* (8) are the rates of the LA supply in arterial blood, the uptake of LA, the formation of 13-HODE, and DNA synthesis (measured here by changes in [^{3}H]thymidine incorporation and DNA content). Each step is dependent on the preceding event so that an increase in arterial blood LA concentration leads to higher steady-state rates of [^{3}H]thymidine incorporation (17, 25) and tumor growth (30, 31) after about 1 and 10 h, respectively. The presence of n-3 FAs in plasma effectively blocked tumor [^{3}H]thymidine incorporation. The addition of 13-HODE to n-3 FA-containing arterial blood restored tumor [^{3}H]thymidine incorporation but had no effect on the inhibited uptake of the saturated, monounsaturated, or n-6 PUFAs. All consecutive reactions were restored when PTX, forskolin, or 8-Br-cAMP was added, indicating that each of the three agents reversed an inhibition at an early stage. The data suggest that the inhibition site is FA uptake, possibly the actual transport of LA and other plasma-saturated, monounsaturated, and n-6 PUFAs across the vascular endothelium and tumor plasma membrane. Because forskolin and 8-Br-cAMP reversed the inhibition by n-3 FAs, cAMP must be required at this early step.

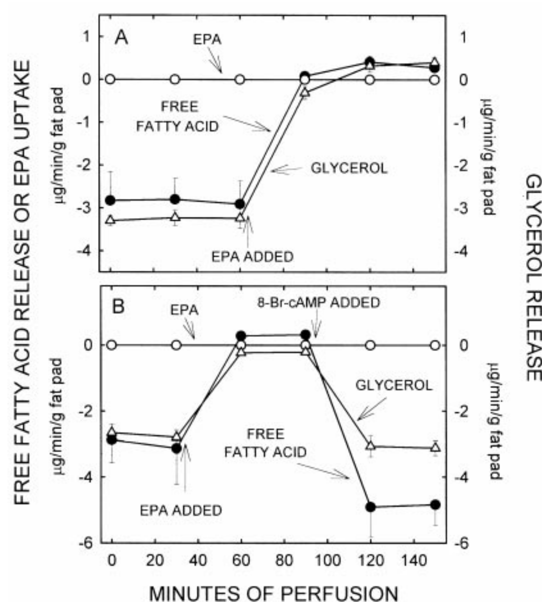

Fig. 4. Effect of EPA (*A*) or EPA followed by 8-Br-cAMP (*B*) on FFA and glycerol release from inguinal fat pads perfused *in situ*. Rats were fasted for 48 h before the experiments. Each point represents the mean ± SD for three perfusions. The arterial blood plasma EPA concentrations in the six control perfusions (○ in *A* and *B*) was <5 μM. The mean arterial blood EPA concentration in *A* (added at 66 min) and *B* (added at 36 min) was 0.78 ± 0.17 mM. Negative values represent FFA and glycerol release from the fat pads. There was no uptake of the added EPA. Mean weight was 5.0 ± 0.3 g (n = 6).

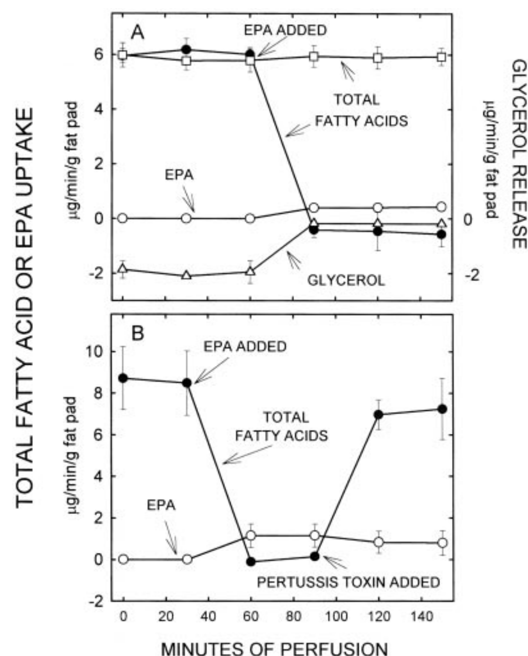

Fig. 5. Effect of EPA (*A*) or EPA followed by PTX (*B*) on uptake of total FA and EPA and release of glycerol from inguinal fat pads in fed rats perfused *in situ*. Each point represents the mean ± SD for three perfusions. The mean arterial blood plasma EPA concentration for the three control perfusions (□ in *A*) was <20 μM. The mean arterial blood plasma:EPA concentration in *A* (added at 66 min) and in *B* (added at 36 min) was 0.84 ± 0.05 and 0.68 ± 0.20 mM, respectively. PTX added in *B* at 96 min was 0.5 μg/ml arterial blood plasma. Mean fat pad weight was 5.2 ± 0.3 g (n = 9).

In a recent report (19), we showed that the neurohormone melatonin inhibited the uptake of saturated, monounsaturated, and n-6 PUFAs and the formation of 13-HODE in hepatoma 7288CTC via a G_i protein-coupled signal transduction pathway. Melatonin acts through cell surface receptors to decrease the intracellular concentration of cAMP (32). The actions of melatonin were reversed by PTX, forskolin, 8-Br-cAMP, and the Servier compound S20928, a melatonin receptor antagonist (33). Compound S20928 had no effect on the inhibition of FA uptake by n-3 FAs (data not shown), indicating that despite the shared postreceptor pathways, melatonin and n-3 FAs are likely to have different receptors. We propose that the decreased LA uptake and 13-HODE release caused by n-3 FAs in hepatoma 7288CTC act via a putative n-3 FA receptor-mediated, G_i protein-coupled signal transduction pathway that decreases intracellular cAMP. This mechanism may explain why LA-dependent growth of many rodent tumors (3, 4, 12) and human breast (9, 10, 16, 34, 35) and prostate (36) cancer xenografts in immunodeficient rodents is inhibited by n-3 FAs. Preliminary experiments indicate that 13-HODE release and [^{3}H]thymidine incorporation in MCF-7 human breast cancer xenografts in nude rats perfused *in situ* are inhibited by melatonin and EPA and that the addition of 13-HODE to the arterial blood restored [^{3}H]thymidine incorporation but not LA uptake.[4] Although 13-HODE is a mitogen in rat hepatoma 7288CTC and in MCF-7 human breast cancer xenografts, it may not be mitogenic in all LA-requiring tumors. There is evidence that 13-HODE, which is formed in normal human colon epithelium (37) and guinea pig epi-

[4] Unpublished results.

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dermis (38), has antiproliferative effects in colon carcinomas (37) and hyperproliferating epidermis (38). It seems that the LA-derived mitogen in neoplastic cells will prove to be tissue specific. It is important to note, however, that n-3 FAs and melatonin blocked the uptake of both plasma LA and arachidonic acid. Therefore, tumor growth that is dependent on a lipid mediator derived from either plasma LA or arachidonic acid will be inhibited.

Other mechanisms were proposed to explain the antitumor actions of n-3 FAs. Competitions between n-3 FAs and LA and arachidonic acid for the enzymes of elongation, desaturation, and arachidonic acid metabolism were proposed to slow tumor growth by decreasing production of growth-enhancing lipid mediators (13, 39, 40). This mechanism does not seem to operate in hepatoma 7288CTC. First, arachidonic acid itself did not stimulate growth in rodent tumors (7), even in essential FA-deficient rats (8, 17). Second, the growth-inhibitory effects of n-3 FAs were reversed by the addition of forskolin, PTX, and 8-Br-cAMP (Fig. 1). n-3 FAs had no effect on 13-HODE formation in the presence of these agents, indicating that the proposed competitions between LA, α-linolenic acid, and EPA did not adversely affect lipoxygenase activity. Also proposed was a decrease in tumor promotion caused by changes in PL n-3:n-6 FA ratios (40) and an increase in the potential for auto-oxidation caused by incorporation of n-3 FAs into tumor membrane lipids (10, 34). We have no evidence that our results were influenced by these reactions. However, they may become important as tumor growth progresses.

The effects of n-3 FAs on FA transport in inguinal fat pads were particularly interesting. In fasted rats, n-3 FAs decreased the rate of release of FFAs and glycerol originating from lipolysis of TAGs to essentially zero (Fig. 4*A*). The release was restored by 8-Br-cAMP (Fig. 4*B*) and by PTX or forskolin (data not shown). This finding confirmed the report of Tisdale and Beck (41), who showed that EPA inhibited lipolysis (as measured by glycerol release) and lowered the intracellular cAMP concentration in isolated murine epididymal adipocytes *in vitro*. In their experiments, the effects of EPA were reversed by PTX. Tisdale (42) and Price and Tisdale (43) suggested that EPA acted through a putative EPA receptor-mediated, G_i protein-coupled pathway. It is unclear at this point whether added 8-Br-cAMP restored FFA and glycerol release from the fat pads by increasing lipase activity, FFA transport, or both. A-V measurements performed across intact inguinal fat pads *in vivo* and during perfusion *in situ* are unable to distinguish between these mechanisms. Hormone-sensitive lipase, the enzyme believed to be responsible for lipolysis in adipose tissue, is activated by phosphorylation by protein kinase A. In a recent report, Osuga *et al.* (44) showed that when both alleles for the hormone-sensitive lipase were disrupted in knockout mice, adipocytes in white adipose tissue were enlarged, but the mice were not obese. White adipose tissue still retained 40% of the TAG lipase activity compared with wild-type unaltered mice. Thus, one or more additional lipases with unknown properties may also contribute to lipolysis in adipocytes.

In fed rats (Fig. 5), uptake of the saturated, monounsaturated, and n-6 PUFAs was inhibited by n-3 FAs in a manner similar to that observed in hepatoma 7288CTC. Utilization of plasma FAs and glucose by inguinal fat pads and their metabolism and storage as TAGs are anabolic reactions mediated by feeding, insulin, and other agents. Intracellular cAMP concentrations are at basal levels in adipose tissue during TAG synthesis (45). This basal level was sufficient to support substantial rates of total FA uptake and a small glycerol release. EPA reduced these rates to about zero, but the pre-EPA rates were restored by 8-Br-cAMP and forskolin. The quantitative relationships between intracellular cAMP concentrations, FFA transport, lipolysis, and other cAMP-regulated reactions are not yet fully understood (45). Presumably, basal levels of cAMP support uptake of the saturated, monounsaturated, and n-6 PUFAs directly by an undefined mechanism; FFA release may require higher intracellular cAMP concentrations. Experiments to determine the effects of n-3 FAs, PTX, and forskolin on the basal levels of cAMP in hepatoma 7288CTC and inguinal fat pads *in vivo* and during perfusion *in situ* are in progress. We suggest that control by n-3 FAs of FFA uptake and release in inguinal fat pads is the likely mechanism for their anticachectic actions to preserve host fat stores in tumor-bearing animals (41–43).

n-3 FAs do not seem to compete directly with the plasma-saturated, monounsaturated, and n-6 PUFAs for entry into hepatoma 7288CTC, as we suggested previously (17). Rather, the evidence indicates that the uptakes of the n-3 FAs and other plasma FAs occur independently of each other. At the plasma n-3 FA concentrations used in these experiments, uptake of the saturated, monounsaturated, and n-6 PUFAs was completely inhibited. Despite this inhibition, n-3 FA uptake continued at measurable rates in hepatoma 7288CTC and inguinal fat pads in fed rats and was less affected by the addition of PTX, forskolin, or 8-Br-cAMP, agents that completely restored uptake of the plasma saturated, monounsaturated, or n-6 PUFAs. Previous experiments showed that the uptake of FAs was directly related to the rate of supply to hepatoma 7288CTC (8, 24) and that the inhibitory the effects of the n-3 FAs were dose dependent (17). The plasma K_i value for inhibition of LA uptake by α-linolenic acid was 0.18 mM, a value that is well within the range of concentrations observed in rodents fed dietary n-3 FAs (11, 14, 46, 47). Therefore, at a given n-3 FA concentration, an increase in the plasma LA concentration will attenuate the negative effect of the n-3 FA. Hudson *et al.* (14) described this interaction between ingested LA and EPA in mice bearing the MAC16 colon adenocarcinoma. Groups of mice fed laboratory chow were given daily oral doses (by gavage) of water, LA, EPA, or EPA + LA. EPA administration increased the serum EPA concentration and reduced the tumor growth rate relative to that of the water- and LA-treated mice. Treatment with EPA + LA increased the plasma LA concentration and the tumor growth rate but did not change the serum EPA concentration.

Understanding the mechanism by which FAs are transferred from the plasma to the cell interior is a critical issue in cancer growth, cachexia, and lipid homeostasis. The mechanism is not yet clearly resolved. Experimental evidence supports two hypotheses: (*a*) that the lipophilic FAs penetrate cell membranes by diffusion (reviewed in Ref. 20); and (*b*) that transport requires specific carriers (reviewed in Ref. 21). Recent findings have strengthened the second hypothesis that specific transporters with high affinity for long-chain FAs are involved. FATP1, a murine membrane protein, was shown to facilitate FA uptake in 3T3-L1 adipocytes (48). The *FATP1* gene is a member of a family of five to six related genes represented among several tissues within a species and with homologues in different species (49). FATP1 mRNA is highly expressed in tissues with high rates of FA metabolism, such as the heart, adipose tissue, skeletal muscle, liver, testis, and intestine (48–50). Relative to normal liver, FATP mRNA seems to be overexpressed in hepatoma 7288CTC (19), suggesting that its expression may be linked to progression. The upstream region of the murine *FATP1* gene contains an insulin response element (51), and expression of FATP1 mRNA levels in 3T3-L1 adipocytes is up-regulated by nutrient depletion and down-regulated by insulin (52). A peroxisome proliferator-activated response element was identified in the murine *FATP1* gene (53). LA, which is a natural ligand for peroxisome proliferator-activated receptors γ and α, up-regulated FATP1 expression (53). The primary sequence of FATP1 has similarities to that of a large family of acyl-CoA-transferases, and evidence was presented that FATP1 has long-chain FA acyl-CoA transferase activity; FA influx may be coupled to esterification (54). It is not yet known whether FA efflux, such as that which occurs during lipolysis, requires FATP1, or whether efflux occurs by diffusion. Given the critical role of cellular FA transport in energy metabolism

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and lipid homeostasis, it seems very likely that additional controls of *FATP1* gene transcription will be found. In this study and in a previous report we have shown that n-3 FAs and melatonin (19) exert important kinetic controls over FA uptake in hepatoma 7288CTC and over FA uptake and release in inguinal fat pads *in vivo* and during perfusion *in situ*. These controls are mediated by well-documented (melatonin) and putative (n-3 FAs) cell surface receptors. The information is transmitted inside the cells via G_i protein-coupled signal transduction pathways and is responsible for the antitumor and anticachectic properties of these agents.

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