

Melatonin Inhibition of Cancer Growth *in Vivo* Involves Suppression of Tumor Fatty Acid Metabolism via Melatonin Receptor-mediated Signal Transduction Events¹

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ABSTRACT

The growth of rat hepatoma 7288CTC *in vivo* is stimulated by the uptake of linoleic acid (LA) and its metabolism to 13-hydroxyoctadecadienoic acid (13-HODE), an important mitogenic signaling molecule within this tumor. Conversely, the growth of a variety of experimental cancers *in vivo* is inhibited by either physiological or pharmacological levels of the pineal gland hormone melatonin, although the mechanism(s) are unknown. We tested the hypothesis that the mechanism of melatonin's anticancer action *in vivo* involves the inhibition of tumor LA uptake and metabolism to 13-HODE in hepatoma 7288CTC. Tumor uptake of LA and release of 13-HODE, measured in tissue-isolated rat hepatoma 7288CTC at 4-h intervals over a 24-h period, were highest during the light phase and lowest during the mid-dark phase, when plasma melatonin levels were lowest and highest, respectively. Pinealectomy eliminated this rhythm of tumor LA uptake and 13-HODE production, indicating that it was driven by the circadian melatonin rhythm. Perfusion of tissue-isolated tumors *in situ* with melatonin (1 nM) rapidly and reversibly inhibited the uptake of plasma fatty acids (FAs), including LA, and its metabolism to 13-HODE. These inhibitory effects of melatonin on tumor FA uptake and 13-HODE release were completely reversed by perfusion of tumors *in situ* with melatonin receptor antagonist S-20928, pertussis toxin, forskolin, or 8-bromo-cAMP. Perfusion of tumors *in situ* with melatonin also decreased tumor [³H]thymidine incorporation and DNA content; these effects on DNA synthesis were also prevented by the coperfusion of tumors with melatonin and S-20928, pertussis toxin, forskolin, 8-Br-cAMP, or 13-HODE. Pinealectomy stimulated tumor growth, LA uptake and metabolism to 13-HODE, and FA storage in hepatoma 7288CTC, whereas melatonin administration (200 µg/day) was inhibitory *in vivo*. Northern blot analysis revealed that, compared with normal liver tissue, hepatoma 7288CTC overexpressed mRNA transcripts for a plasma membrane-associated FA transport protein (FATP). FATP mRNA expression was unaffected by the treatment of tumor-bearing rats with daily afternoon melatonin injections or exposure to constant light. These results support a novel mechanism of tumor growth inhibition by melatonin involving a melatonin receptor-mediated suppression of cAMP levels, resulting in diminished tumor FA transport, possibly via decreased FATP function. The inhibition of these signal transduction events by melatonin culminates in the suppression of LA uptake, LA metabolism to the mitogenic signaling molecule 13-HODE, and cancer growth.

INTRODUCTION

Adequate supplies of dietary LA⁴ and energy are required for optimal growth of solid rodent tumors *in vivo* (1, 2). Studies from our laboratory using tissue-isolated hepatoma 7288CTC show that augmented arterial blood plasma concentrations of LA, resulting from either increased dietary intake or mobilization of host fat stores, are taken-up by this tumor and exert a direct stimulative effect on tumor growth and metabolism *in vivo* (3–7). A portion of the LA taken up by hepatoma 7288CTC is converted to 13-HODE (8). In a companion paper in this issue of *Cancer Research* (9), we show that 13-HODE formed in this tumor, via lipoxygenase activity, is the mitogenic signal for LA-dependent growth of hepatoma 7288CTC. As judged from the recent study of Glasgow *et al.* (10), 13-HODE enhances EGF-responsive mitogenesis through EGF receptor autophosphorylation and tyrosine phosphorylation of key downstream signal transduction proteins such as MAPK. Several studies support a role for 13-HODE in cell proliferation *in vivo* (8, 9) and *in vitro* (10, 11), but there is evidence that 13-HODE is antiproliferative in certain other target tissues (12).

The pineal gland exhibits a prominent circadian rhythm in its production and release of melatonin into the bloodstream during darkness (13, 14). Melatonin plays an important role in a number of physiological and pathophysiological processes, including circadian rhythm regulation, seasonal reproduction (13, 14) immune function (15), and tumorigenesis (16). In experimental models of neoplasia, melatonin inhibits tumorigenesis in an apparently circadian time-dependent manner (16–20), and evidence from a number of clinical cancer trials suggests that it may be an effective therapeutic agent, either alone or in combination with standard anticancer treatments (21). Although some progress has been made in elucidating the mechanisms of melatonin's oncostatic action *in vitro* (22–25), no definitive mechanism(s) have emerged to explain the anticancer effects of melatonin in the more relevant *in vivo* setting.

A limited number of investigations in rodents have suggested that melatonin may also exert a modulatory role on normal lipid metabolism, depending on the species studied and the experimental conditions used (26). In relation to tumor growth, a potentially important link between nutritional factors and melatonin was first suggested by the observation that melatonin's oncostatic effect is accentuated in energy-restricted animals, as compared with those fed *ad libitum* (27). Energy restriction causes a severe decrease in the plasma LA concentration of the tumor-bearing host (8). In a recent report (28), we demonstrated that an inhibition of melatonin production increased the rates of tumor growth, LA uptake, and 13-HODE release in hepatoma 7288CTC *in vivo*, suggesting the hypothesis that melatonin inhibits tumor growth by suppressing tumor LA uptake and metabolism to 13-HODE. Here, we describe experiments designed to test this hy-

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⁴ The abbreviations used are: LA, linoleic acid; 13-HODE, 13-hydroxyoctadecadienoic acid; EGF, epidermal growth factor; MAPK, mitogen-activated protein kinase; FA, fatty acid; 12L: 12D, 12 h light/12 h dark; PTX, pertussis toxin; FATP, FA transport protein; HPLC, high-performance liquid chromatography.

pothesis and elucidate the mechanism of these interactions. The results show that melatonin inhibits tumor growth, LA uptake, and metabolism to 13-HODE through signal transduction events encompassing G protein-coupled melatonin receptor suppression of cAMP (29).

MATERIALS AND METHODS

Animals, Diets, and Tumor Implantation and Growth. Male Buffalo rats BUF(BUF/Ncr)(NCI) rats (4–5 weeks old) weighing 35–75 g were purchased from Charles River Laboratories (Kingston, NY). The care and use of the animals used in our experiments were in accordance with the guidelines of our Institutional Animal Care and Use Committee. Male Sprague Dawley rats weighing 150–200 g were purchased from Harlan Sprague Dawley (Indianapolis, IN). Buffalo rats were given free access to water and a semipurified diet containing 5% corn oil to provide a fixed amount of LA. Diets were prepared weekly and contained 0.5 g of BHT (Sigma Chemical, St. Louis, MO) per 1500 g of diet and were stored in sealed plastic bags at -20°C . Rats were housed two per cage and obtained their food from wide-mouthed glass feeding jars. Sprague Dawley rats, used as blood donors for the perfusion experiments, were housed two per cage and given free access to water and a standard laboratory diet (Prolab mouse, rat, and hamster 1000 formula; Agway Inc., Syracuse, NY). All rats were subjected to alternate 12-h periods of dark and light (lights on 6:00 a.m.–6:00 p.m.). There was no light contamination in the animals rooms during the dark phase (28), and the temperature and humidity were maintained at 23°C and 40–60%, respectively. In one experiment, tumor-bearing rats were maintained on constant light (800 lux) 24 h per day.

Procedures for implantation of tissue-isolated Morris hepatoma 7288CTC in male Buffalo rats were described in detail in previous reports (3–8) and in the companion paper (9). Briefly, a 3-mm cube of tumors was sutured to the tip of a vascular stalk formed from the superficial epigastric artery and vein. The tumor 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; subsequent tumor growth was s.c. The latency period (*i.e.*, time to first palpable, pea-sized mass) was determined for each animal in each experiment. When tumors reached sufficient size for measurement, rats were subjected to light CO_2 narcosis, and tumor dimensions were measured through the skin with vernier calipers and were converted to tumor weights as described previously (3). Growth rates (g/day) were generated by linear regression from the estimated tumor weights for the treated and control animals during the course of the study. The final tumor weight was determined by weighing at the end of the experiment.

Measurement of Tumor FA Uptake and 13-HODE Release *in Vivo* Over a 24-h Period. At 12 weeks of age, adult male Buffalo rats were implanted with hepatoma 7288CTC, as described above. When estimated tumor weights were ~ 5 g, the rats were anesthetized and anticoagulated (6, 8). The carotid artery and tumor vein were cannulated, and blood samples were collected from each vessel simultaneously. Measurements of arteriovenous differences for total FAs, LA, and 13-HODE were performed, and rates of uptake and release were calculated as described previously (8). Blood collections were performed on separate groups of animals (four/group) sampled every 4 h during a 24-h period. Plasma melatonin levels were also immunoassayed at these timepoints (30). The effects of removal of the nocturnal melatonin signal on the rhythm of tumor FA metabolism were determined in a separate experiment. Male Buffalo rats maintained on 12L:12D were anesthetized with pentobarbital (25 mg/kg body weight) and either pinealectomized or sham-operated prior to tumor-implantation as described previously (31). When tumors in these rats reached an estimated weight of 5 g, blood samples for arteriovenous difference measurements for total FAs, LA, and 13-HODE were collected either during the light phase (8:00 a.m.) or dark phase (12:00 a.m.). For collection of the dark phase samples, animals were anesthetized in the dark room under a dim red light (Kodak safelight, Eastman Kodak Co., Rochester, NY), and their eyes were covered with black electrical tape. With their eyes completely shielded from light, the animals were transferred to an adjoining darkened room, and arteriovenous difference measurements were performed as described above. Illumination from the dissecting microscope provided the only source of light.

Perfusion of Tissue-isolated Hepatoma 7288CTC *in Situ* with Melatonin, Alone or in Combination with Melatonin Receptor Antagonist S-20928, PTX, Forskolin, 8-bromo-cAMP, or 13-HODE: Measurements of LA Uptake, 13-HODE Release, DNA Content, and [^3H]Thymidine Incorporation into DNA. Tumors weighing ~ 5 g in male Buffalo rats, were perfused *in situ* as described (Refs. 4, 5, and 7; see Ref. 9 for details). Essentially melatonin-free arterial blood was collected during the early light phase (8:15–8:45 a.m.) from donor male Sprague Dawley rats. Tumor-bearing rats were fasted for 48 h to increase in plasma lipids. Donor blood was contained in a chilled (4°C) reservoir and was pumped to the tumor through a warm water bath (37°C) and an artificial lung. A 30-min preperfusion period was used to allow the establishment of steady-state conditions prior to sample collection. After collection of the zero time samples, tumors were perfused for 65 min before melatonin (Regis Chemical Co., Morton Grove, IL) was added to the reservoir arterial blood. The melatonin addition was sufficient to give a whole blood concentration of ~ 1 nM; the actual plasma melatonin concentrations, measured by radioimmunoassay, were 2–4 nM (32). The transit time for the melatonin-containing perfusate to reach the tumor from the reservoir was ~ 20 min. Tumor perfusion continued for an additional 95 min. To determine whether the effects of melatonin were reversible, perfusions were started with blood containing melatonin (1 nM). After collection of samples at 60 min, the arterial blood supply to the tumor was switched to a separate reservoir containing melatonin-free blood, and the perfusion was continued for an additional 95 min. The effects of several agents were tested on the tumor response to melatonin. These perfusions were performed as follows: tumors were perfused for 30 min with whole blood (no exogenous melatonin), melatonin (1 nM) was added to the reservoir, and the perfusion was continued for 60 min more. At 95 min, melatonin receptor antagonist S-20928 (1 nM; Servier, Courbevoie Cedex, France), PTX (0.5 $\mu\text{g}/\text{ml}$), forskolin (1 μM), 8-bromo-cAMP (10 μM), G protein antagonist NF-023 (0.5 $\mu\text{g}/\text{ml}$; Calbiochem-Novabiochem, La Jolla CA), 13-HODE (12 $\mu\text{g}/\text{ml}$), or vehicle was added, and the perfusion was continued for an additional 60 min. Total perfusion times were typically 120–150 min.

Arteriovenous differences for total FAs, LA, 13-HODE, hematocrit, pO_2 , pCO_2 , and pH were measured as described previously (5, 6, 9). In some experiments, tumor glucose uptake and lactate release were also measured (4). Incorporation of [^3H]thymidine into tumor DNA was initiated 20 min before the end of the experiment by injection of 20 μl of physiological saline containing 2 μCi [methyl- ^3H]thymidine/g estimated tumor weight into the arterial catheter immediately before the tumor. The [^3H]thymidine made one pass through the tumor; unincorporated radioactivity was washed out of the perfused tumor during the remaining 20 min of perfusion. Radioactivity incorporated into tumor DNA was measured by liquid scintillation using internal standardization and is reported as dpm/ μg tumor DNA (4, 9). Tumor DNA content was measured in 20% (w/w) homogenates fluorometrically using Hoechst dye 33258 using the procedure described in Technical Bulletin 119 (Hoefler Scientific Instruments, San Francisco, CA).

Tumor Growth and FA Metabolism *in Vivo* in Response to Melatonin or Pinealectomy. Melatonin (200 $\mu\text{g}/0.1$ ml of 0.02% ethanolic saline) and vehicle injections (0.1 ml of 0.02% ethanolic saline) were administered s.c. daily in the late afternoon (4:00–6:00 p.m.) to rats maintained on a semipurified diet containing 5% corn oil *ad libitum*. The late afternoon administration of melatonin has been shown to be the optimal circadian time for this indole to produce a tumor growth-inhibitory effect *in vivo* (17). In the dietary study, melatonin was mixed with the semipurified diet so that each rat consumed ~ 200 μg of melatonin per day. Control animals were maintained on a melatonin-free 5% corn oil diet. In the pinealectomy study, rats maintained on a 12L:12D cycle and provided with a standard laboratory diet were either pinealectomized or sham-operated (31). Melatonin injections or feeding as well as pinealectomy and sham-pinealectomy procedures were initiated or performed 1 week prior to the implantation of tumor tissue. Melatonin injections or feeding were continued until the end of each growth experiment. Following collection of blood samples for arteriovenous difference measurements, the tumors were excised, weighed, snap-frozen in liquid N_2 , and stored at -80°C until analysis for mRNA (see below).

Tumor Expression of FATP mRNA. Total RNA was extracted using TRI-reagent (Molecular Research Center, Cincinnati, OH) from snap-frozen tissues pulverized under liquid nitrogen. Northern blots were performed with 15 μg RNA per sample. FATP (34, 35)-specific riboprobes were made with

plasma pCR1000 (Invitrogen, Carlsbad, CA) containing a 1.087-kb fragment of the FATP open reading frame (coordinates 207–1293). (Ref. 33). Probes were labeled with [α - 32 P]UTP (800 Ci/mmol; ICN Biomedicals, Costa Mesa, CA) to a specific activity of at least 5×10^8 cpm/ μ g. To control for RNA loading, we also probed Northern blots with glyceraldehyde 3-phosphate dehydrogenase riboprobes (Ambion Inc., Austin, TX). Hybridization was carried out at 65°C, and the blots were washed at high stringency (68°C, 0.1 \times SSC-0.1% SDS) and exposed to Kodak BIOMAX MS x-ray film with a BIOMAX MS intensifying screen at -70°C. FATP mRNA were normalized to the glyceraldehyde 3-phosphate dehydrogenase signals following densitometry of the X-ray films.

Lipid Extraction and Analyses. Total plasma, tumor, and dietary lipids were extracted using the procedure of Folch *et al.* (36) and analyzed by gas chromatography, as described previously (5, 8). Extraction of 13-HODE from tumor venous blood and analysis by HPLC were performed as described (8). Details of these procedures are described in the companion report (9).

Statistical Analysis. Differences among mean tumor total FA and LA uptake, tumor 13-HODE release, and plasma melatonin levels over a 24-h period were determined with a one-way ANOVA followed by the Student-Neuman-Keul *post hoc* test. Differences between the mean latency to tumor palpability between experimental groups and their corresponding control groups were determined by Student's *t* test. Differences between the slopes of regression lines between experimental groups and their corresponding control groups were determined by regression analyses and tests for parallelism (Student's *t* test). All statistical analyses were performed using the statistical software package TRUE EPISTAT (Epistat Services, Richardson, TX). Differences were considered to be statistically significant at $P < 0.05$.

Materials. Chloroform (HPLC grade), ethyl acetate, methanol, glacial acetic acid, heptane, hexane, and Sep-Pak C18 Cartridges for HPLC sample extraction were purchased from Fisher Chemical Co. (Pittsburgh, PA). Methyl esters of rapeseed oil and FA HPLC standards were obtained from Supelco (Bellefonte, PA). Free FA and lactic acid standards, BHT, boron trifluoride-methanol, enzymes, nucleotides, and buffers were purchased from Sigma Chemical Co. (St. Louis, MO). The HPLC standards, 5-HETE and 13-HODE (each as the racemic mixture) were purchased from Cayman Chemical Co. (Ann Arbor, MI). Dietary ingredients were purchased from United States Biochemical (Cleveland, OH).

RESULTS AND DISCUSSION

Melatonin-driven Circadian Rhythm of LA Uptake and Metabolism to 13-HODE by Hepatoma 7288CTC. As a first step, we determined the relationship between the physiological, endogenous melatonin signal and tumor LA uptake and metabolism to 13-HODE. This was of particular interest because plasma FA levels are elevated during the dark phase when food intake is highest (6, 28). As shown in Fig. 1, hepatoma 7288CTC exhibited a rhythm of LA uptake and metabolism to 13-HODE over a 24-h period that was temporally correlated with the circadian melatonin rhythm. Total FA (Fig. 1A) and LA (Fig. 1B) uptake and 13-HODE release (Fig. 1C) were highest during the light phase of an alternating light/dark cycle when plasma melatonin levels were lowest (Fig. 1D). During the middle of the dark phase (12:00 a.m.), when plasma levels of melatonin were at their peak, FA uptake was 60–70% lower than uptake during the light phase, whereas 13-HODE release was undetectable. Thus, in hepatoma 7288CTC the circadian rhythm of LA uptake and metabolism to 13-HODE was the reverse of the melatonin rhythm, indicating that the light/dark rhythm of tumor FA uptake and metabolism was not an endogenous tumor rhythm but one that was passively driven by the circadian melatonin rhythm. Additionally, Fig. 2, which depicts the relationship between endogenous plasma melatonin concentrations and LA uptake and 13-HODE production by hepatoma 7288CTC, indicates that the inhibition of LA uptake and metabolism to 13-HODE is inversely related to endogenous circulation melatonin levels.

Further evidence that the light/dark rhythm of tumor FA uptake and metabolism was driven by the circadian melatonin rhythm was ob-

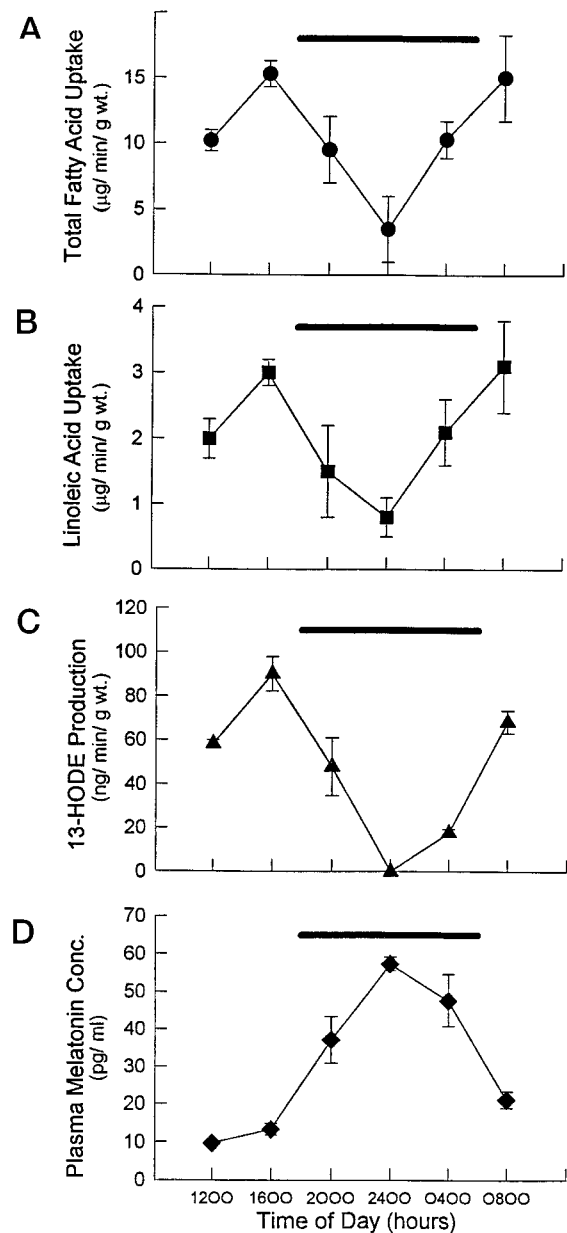


Fig. 1. Mean tumor uptake of total FAs (A) and LA (B), release of 13-HODE by hepatoma 7288CTC *in vivo* (C), and plasma melatonin levels over a 24-h period (D) in adult male Buffalo rats. Data points means; horizontal bars, SE. Animals were maintained on a 12L:12D cycle and provided with a semipurified diet containing 5% corn oil *ad libitum*. Tumor arteriovenous difference measurements were made over 4 h during a 24-h period. Tumor-bearing rats ($n = 4$ per time point) were randomized, such that the mean (\pm SD) tumor weight at each time point was 5.4 ± 0.1 g. The dark bar at the top of each graph represents the duration of the dark phase. A, tumor total FA uptake at 12:00 a.m. versus 4:00 a.m., 8:00 a.m. or 4:00 p.m. ($P < 0.05$). B, tumor LA uptake at 12:00 a.m. versus 8:00 a.m. and 4:00 p.m. ($P < 0.05$). C, 13-HODE release at 12:00 a.m. versus 8:00 a.m., 12:00 p.m., 4:00 p.m., and 8:00 p.m. ($P < 0.05$). D, plasma melatonin levels at 12:00 a.m. versus 8:00 a.m., 12:00 p.m., 4:00 p.m., and 8:00 p.m. ($P < 0.05$).

tained from measurements at single time points near the beginning of the light phase (8:00 a.m.) and during the mid-dark phase (12:00 a.m.) in pinealectomized, sham-operated, or intact rats maintained on a 12L:12D cycle. The data in the sham-operated and intact groups were virtually identical and were combined into one group, designated the sham/intact group. As expected from our previous studies (6, 28), the arterial concentration and, thus, the supply of total FA to the tumors was 2-fold higher during the dark phase in all the treatment groups (Table 1) as a result of an intact circadian rhythm of food intake. As

observed in the circadian study above, total FA and LA uptake was decreased by 80–85% and 13-HODE release was abolished in tumors in sham/intact rats during the dark phase (Table 1). However, these effects seen in sham/intact rats were completely negated in pinealectomized animals. In fact, pinealectomy caused a 10–16-fold elevation in total FA and LA uptake and a 200-fold higher rate of 13-HODE release, as compared with values in the sham/intact controls during darkness (Table 1). Therefore, the lack of suppression of tumor FA uptake and metabolism during the dark phase in pinealectomized rats (*i.e.*, no melatonin rhythm) indicates that the tumor rhythm of lipid metabolism shown in Fig. 1 was driven by the nocturnal inhibitory melatonin signal.

Effects of Perfusion of Tissue-isolated Hepatoma 7288CTC *in Situ* with Melatonin on LA Uptake and Metabolism. Because the circadian melatonin signal was responsible for the nocturnal suppression of tumor LA uptake and metabolism to 13-HODE, we evaluated the direct effects of a physiological nocturnal melatonin concentration (1 nM) on tissue-isolated tumors perfused *in situ*. Fig. 3A (*left*) shows that the addition of exogenous melatonin to the whole blood perfusate, collected from fasted rats during the early light phase, caused a 70% decrease in total FA and LA uptakes. Similarly, 13-HODE release fell to an undetectable level during perfusion with melatonin. The suppression of total FA and LA uptake and 13-HODE release by melatonin was sustained for 50–60 min, until the end of the perfusion. Removal of melatonin from the perfusate reversed the suppressive effects on FA uptake and 13-HODE release (Fig. 3A, *right*). The rapid decrease in 13-HODE release in response to melatonin was most likely due to melatonin-induced inhibition of tumor LA uptake. However, an additional inhibitory action of melatonin on lipoxygenase activity could potentially have contributed to the disappearance of 13-HODE from the venous effluent. Neither melatonin's immediate precursor, *N*-acetylserotonin, nor its major liver metabolite, 6-hydroxymelatonin (13, 14), affected tumor FA uptake or metabolism

Table 1 Total arterial fatty acid concentration, total FA and LA uptake and 13-HODE release in tissue-isolated hepatoma 7288CTC during the light phase (8:00 a.m.) or dark phase (12:00 a.m.) in either pinealectomized or sham-operated/intact male Buffalo rats maintained on a 12L:12D cycle (lights on 6:00 a.m.–6:00 p.m.)

Values are means \pm SD. Data in sham-operated and intact animals were not statistically different and were combined.

Treatment	Arterial plasma total FAs (mg/ml)	Total FA uptake (μ g/min/g)	LA uptake (μ g/min/g)	13-HODE release (ng/min/g)
Light phase (8:00 a.m.)				
Sham/intact (6) ^a	1.3 \pm 0.2	7.7 \pm 1.2	1.6 \pm 0.4	35.9 \pm 5.6
Pinealectomized (5)	1.2 \pm 0.2	9.1 \pm 1.8	1.7 \pm 0.4	31.3 \pm 3.4
Dark phase (12:00 a.m.)				
Sham/intact (9)	2.6 \pm 0.3 ^b	1.2 \pm 1.8 ^c	0.3 \pm 0.4 ^c	ND ^d
Pinealectomized (7)	2.5 \pm 0.3 ^a	16.9 \pm 3.9 ^e	3.3 \pm 1.2 ^e	209.3 \pm 18.5 ^e

^a Numbers in parentheses indicate the number of animals/tumors per group.

^b $P < 0.05$ vs. light phase sham/intact and pinealectomized groups.

^c $P < 0.05$ vs. light phase sham/intact and dark phase pinealectomized groups.

^d ND, not detectable.

^e $P < 0.05$ vs. light phase pinealectomized group.

(data not shown). Thus, the reversible effects on tumor FA uptake and metabolism were specific for melatonin. Fig. 3 also shows that the melatonin-induced blockade of FA uptake and metabolism did not affect tumor blood flow, glucose uptake, or lactate production (Fig. 3B) or the tumor venous blood pH or arteriovenous differences for pO₂ and pCO₂ (Fig. 3C). We have obtained virtually identical results in tissue-isolated *N*-nitroso, *N*-methylurea-induced rat mammary tumors in female Buffalo rats and MCF-7 human breast cancer xenografts in female nude rats perfused *in situ* with physiological melatonin.⁵ These results clearly show that a physiological nocturnal concentration of melatonin was able to inhibit LA uptake and 13-HODE production in hepatoma 7288CTC directly, rapidly, reversibly, and specifically. We conclude that the melatonin signal is directly responsible for the nocturnal suppression of tumor LA uptake and metabolism to 13-HODE.

Effects of Melatonin Receptor Antagonist S-20298, PTX, Forskolin, and 8-bromo-cAMP on Melatonin Action, LA Uptake, and Metabolism in Tissue-isolated Hepatoma 7288CTC Perfused *in Situ*. Melatonin binding has been documented in neoplastic tissues (16, 22). Elevated levels of cAMP also have been found in several types of malignancies, including hepatoma, melanoma, and mammary carcinoma (22, 37, 38). The involvement of melatonin receptors, which are negatively coupled to adenylate cyclase via a PTX-sensitive G protein (39), in the inhibition of LA uptake and metabolism would require functional linkage to a facilitated transport protein such as FATP (34). Thus, if the melatonin-induced blockade of LA uptake and metabolism is mediated by a suppression of cAMP, a melatonin receptor antagonist S-20928 (40), PTX, forskolin, and cAMP should reverse this effect. Fig. 4 shows that all of these agents completely reversed the melatonin-induced blockade of total FA and LA uptake and 13-HODE release in the perfused tumor. NF-023, a G_i antagonist, had an identical effect on melatonin inhibition of LA uptake and metabolism (data not shown). These findings provide strong evidence for a melatonin receptor-mediated suppression of cAMP production as the mechanism for melatonin's inhibition of LA uptake and metabolism to 13-HODE.

Effects of Melatonin Alone or in Combination with either Melatonin Receptor Antagonist S-20928, PTX, Forskolin, 8-bromo-cAMP, or 13-HODE on DNA Content, [³H]Thymidine Incorporation into DNA, LA Uptake, and 13-HODE Release in Hepatoma 7288CTC Perfused *in Situ*. In the companion report (9), we demonstrated that 13-HODE was the mitogen responsible for LA-dependent tumor growth. Data presented in Fig. 3 indicated that melatonin

⁵ D. E. Blask, R. T. Dauchy, L. A. Sauer, and F. Zalatan, unpublished results.

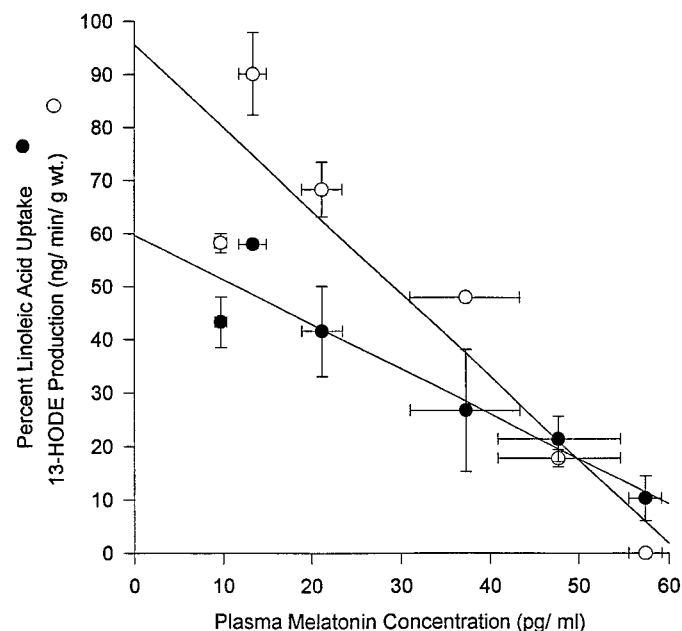
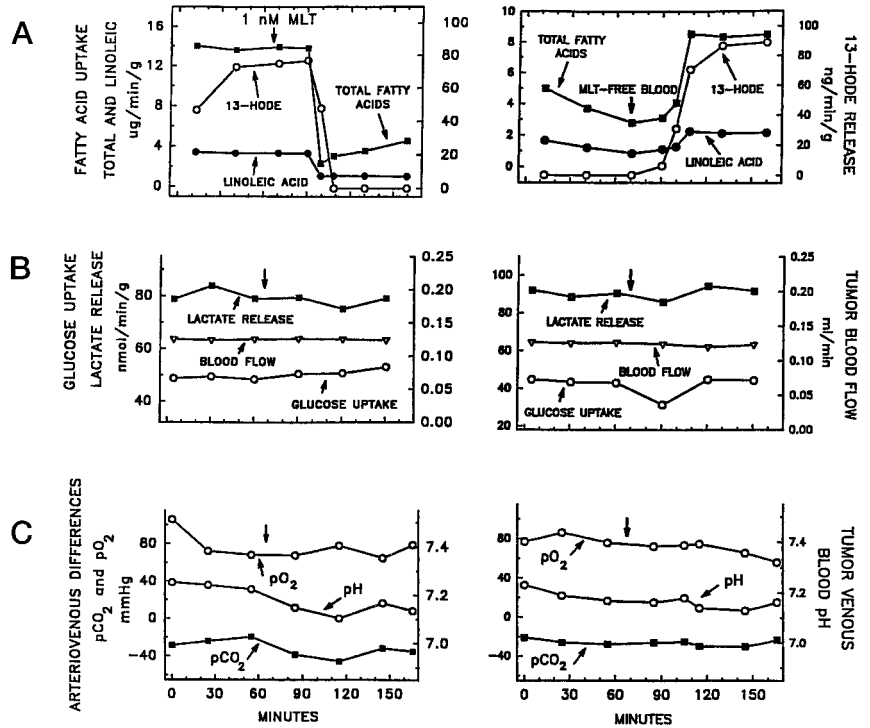


Fig. 2. Data points, mean (vertical bars, SE) tumor LA uptake (%) and 13-HODE production at six different circadian time points over a 24-h period, plotted as a function of increasing mean (horizontal bars, SE) plasma melatonin concentrations measured at the same time points. Data points were derived from the results depicted in Fig. 1. Regression analysis revealed that both LA uptake ($r = -0.9437$) and 13-HODE production ($r = -0.9146$) were inversely correlated with plasma melatonin concentrations ($P < 0.05$). The slopes of the regression lines were not significantly different from each other.

Fig. 3. The effects of melatonin (MLT) addition to whole blood perfusate on tumor uptake of total FA and LA from arterial blood and the release of 13-HODE into tumor venous blood were in a tissue-isolated hepatoma 7288CTC perfused *in situ*. *Left*, a tumor weighing 5 g was perfused *in situ* with blood. After collection of the 60-min sample, melatonin was added (65 min) to the reservoir of donor blood to a final concentration of 1 nM, and the perfusion was continued for an additional 95 min. Approximately 20 min were required for the melatonin-containing perfusate to reach the tumor from the reservoir. The perfusion profile depicted here represents 1 of 10 perfusions that yielded essentially identical results. *Right*, the effects of melatonin removal on tumor (5 g) uptake of total FAs and LA and 13-HODE release in hepatoma 7288CTC perfused *in situ*. The perfusion began with donor blood containing exogenous melatonin (1 nM). After collection of the 60-min sample, the arterial line was switched to a separate reservoir containing donor blood without exogenous melatonin. The perfusion was continued for an additional 95 min. Glucose, lactate, pH, pO₂, pCO₂, and flow rates were monitored in arterial and tumor venous blood throughout the perfusions. The whole blood perfusates in both experiments were collected during the early light phase (8:15–8:45 a.m.) from donor rats fasted for 48 h.



inhibited tumor LA uptake and conversion to 13-HODE, and Fig. 4 shows that the effects of melatonin on 13-HODE release may be reversed by several agents that block signal transduction events, leading to suppression of cAMP production. Therefore, we determined whether the melatonin inhibitory effect on LA uptake and 13-HODE release affected tumor DNA content and the rate of [³H]thymidine incorporation into DNA in the same way as lipoxigenase inhibitors or removal of LA from the arterial blood (9). Table 2 shows that melatonin inhibited LA uptake and 13-HODE release, as expected, and also caused a significant 33% decrease in tumor DNA content and a 43% reduction in [³H]thymidine incorporation into

tumor DNA. Perfusions containing melatonin and S-20928, PTX, forskolin, or 8-bromo-cAMP reversed the melatonin-induced blockage of LA uptake and 13-HODE release and restored both tumor DNA content and the rate of [³H]thymidine incorporation to control values. Of particular importance was the finding that the cop perfusion of melatonin plus 13-HODE blocked the inhibitory effect of melatonin and markedly increased tumor DNA content and [³H]thymidine incorporation by 2- and 10-fold over the controls, respectively; LA uptake remained totally suppressed. The tumor uptake of 13-HODE perfused into the artery was ~53% and was not affected by cop perfusion with melatonin, suggesting that the uptakes of LA and 13-HODE

Fig. 4. Effects of melatonin receptor antagonist S-20928 (A), PTX (B), forskolin (C), and 8-Br-cAMP (D) on melatonin-induced inhibition of FA uptake and 13-HODE release by hepatoma 7288CTC perfused *in situ*. Following a control perfusion period of ~30 min with donor blood, melatonin was added to the reservoir containing the perfusate to give a final concentration of 1 nM. The perfusion with melatonin was continued for an additional 60 min. At 90 min, S-20928 (1 nM), PTX (0.5 μg/ml), forskolin (1 μM), or 8-Br-cAMP (10 μM) was added, and the perfusion continued with these agents combined with melatonin for 60 min. The data reflect the results of four separate perfusions of four different hepatomas. Each of the perfusion profiles depicted here represents one of six perfusions that yielded essentially identical results. Tumor blood flow, pH, pO₂, and pCO₂ were monitored (data not shown) and were not different from the results observed in Fig. 3.

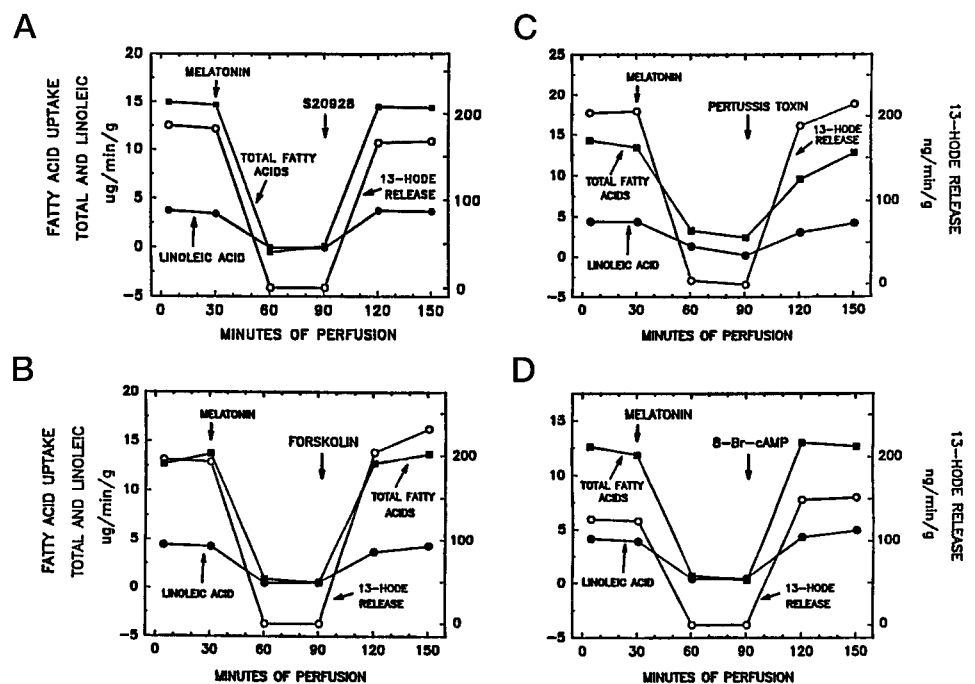


Table 2 Effects of perfusion of tissue-isolated hepatoma 7288CTC with melatonin (1 nM), either alone or in combination with either the melatonin receptor antagonist S20928 (1 nM), PTX (0.5 μg/ml), forskolin (1 μM), 8-Br-cAMP (10 μM), 13-HODE (12 μg/ml), or vehicle on tumor DNA content, [³H]thymidine incorporation into DNA, LA uptake, and 13-HODE release

Values are means ± SD.

Treatment ^a	DNA content (mg/g)	[³ H]thymidine incorporation (dpm/μg DNA)	LA uptake (μg/min/g)	13-HODE (ng/min/g)	
				Arterial supply	Venous output
Control ^b	4.6 ± 0.2	42.1 ± 5.2	1.81 ± 0.13	ND ^c	30.7 ± 1.9
Melatonin	3.1 ± 0.4 ^d	24.1 ± 3.1 ^d	0.43 ± 0.33 ^d	ND	ND
Melatonin + S20928	4.3 ± 0.3	42.9 ± 2.1	1.80 ± 0.18	ND	36.5 ± 0.8
Melatonin + PTX	4.1 ± 0.3	44.9 ± 3.5	1.85 ± 0.23	ND	29.7 ± 4.5
Melatonin + Forskolin	4.3 ± 0.4	41.0 ± 7.5	1.63 ± 0.16	ND	29.7 ± 3.6
Melatonin + 8-Br-cAMP	4.7 ± 0.2	54.8 ± 1.3	2.42 ± 0.29	ND	39.6 ± 3.2
Melatonin + 13-HODE	7.5 ± 0.4 ^e	470.4 ± 31.3 ^e	ND	530.5 ± 52.1	261.8 ± 29.7
13-HODE ^f	7.5 ± 0.4 ^e	448.1 ± 46.1 ^e	2.3 ± 0.34	536.2 ± 75.0	248.0 ± 32.5

^a There were three animals (tumors) per group; mean (± SD) tumor weight = 5.7 ± 0.2 g.

^b Values for the control and 13-HODE-only groups are derived from Fig. 2 of the companion publication (9).

^c ND, not detectable.

^d $P < 0.05$ vs. control and all other melatonin-treated groups.

^e $P < 0.05$ vs. control group.

^f Tumors removed 53% of the 13-HODE.

proceed by different mechanisms. Perfusion of tumors with 13-HODE alone had no effect on LA uptake, indicating that 13-HODE was responsible for the increase in DNA content and [³H]thymidine incorporation in either the presence or absence of melatonin. These results provide unambiguously strong evidence that melatonin's inhibition of hepatoma 7288CTC growth *in vivo* (see below) is ultimately the result rather than the cause of a melatonin-induced suppression of the production/release of the mitogenic signaling molecule 13-HODE via melatonin receptor-mediated signal transduction events.

Effects of Melatonin Treatment of Pinealectomy on the Growth of and LA Uptake and Metabolism by Hepatoma 7288CTC. In view of melatonin's ability to directly suppress tumor LA uptake and 13-HODE production and the mitogenic effects of 13-HODE, we tested whether chronic melatonin treatment, either by daily injections or dietary administration, or chronic elimination of the endogenous melatonin signal by pinealectomy would alter tumor growth in a manner consistent with melatonin-induced alterations in LA uptake, 13-HODE production, and mitogenicity discussed above (Figs. 3 and 4 and Table 2). Melatonin (200 μg) administered either as single daily, late afternoon s.c. injections or provided in the diet caused a 2-fold delay in the mean latency to tumor palpability as follows: melatonin injections (12.0 ± 0.9 days) *versus* saline injections (6.7 ± 0.7 days; $P < 0.05$); dietary melatonin (15.0 ± 0.0 days) *versus* a melatonin-free diet (8.3 ± 0.3 days; $P < 0.05$). Parenteral or dietary melatonin treatment also suppressed the tumor growth rate by 59 and 67%, respectively, as compared with the tumor growth rate in control rats either injected with vehicle or fed a melatonin-free diet ($P < 0.05$; Fig. 5, *top* and *middle*). Arteriovenous difference measurements performed across each tumor in the six rat groups shown in Fig. 5 indicated that tumor LA and total FA uptake and tumor FA content were significantly reduced (48–58%, $P < 0.05$) and that 13-HODE release was almost completely inhibited in melatonin-injected rats (Table 3). Dietary melatonin had a more potent effect and caused a 71–75% reduction in tumor LA and total FA uptake and FA content. The total blockade of 13-HODE release by dietary melatonin was similar to that observed with melatonin injections.

In contrast to the effect of exogenous melatonin, elimination of the nocturnal melatonin signal by pinealectomy substantially decreased the mean latency to tumor palpability as follows: pinealectomy (6.9 ± 0.9 days) *versus* sham operation (14.0 ± 0.5 days; $P < 0.05$). Additionally, pinealectomy increased the tumor growth rate in rats fed a stock diet by 2-fold, as compared with sham-pinealectomized animals (Fig. 5, *bottom*). Because there were no differences in food consumption among any of the experimental and control groups (data

not shown), changes in tumor growth rates and latency to tumor palpability in the melatonin-treated or pinealectomized rats could not be ascribed to alterations in food intake (41). Arteriovenous difference measurements made at the end of the growth period demonstrated that pinealectomy caused a significant 1.4–7.6-fold increase in tumor LA and total FA uptake and FA content and also augmented tumor 13-HODE release by 6.3-fold over that in sham-operated rats ($P < 0.05$; Table 3). The stimulatory effects of pinealectomy on tumor LA uptake, 13-HODE release, and growth closely reproduced the stimulatory effects of constant light or dim light contamination during the dark phase on tumor metabolism and growth previously reported by us (28). These results, together with those of our previous study (28), indicate that the nocturnal melatonin peak encodes a critical physiological oncostatic signal to this tumor and is an important component of a complex array of tumor growth regulatory mechanisms.

Expression of mRNA Transcripts for FATP by Hepatoma 7288CTC. The abrupt inhibition of FA uptake by melatonin in hepatoma 7288CTC and in rat and human mammary cancers does not support models for FA uptake based on simple diffusion through the cell membrane. Reversal of the melatonin inhibition by melatonin receptor antagonist S-20928, PTX, forskolin, 8-bromo-cAMP, and 13-HODE also suggests a specific FA transport carrier. Mechanisms for cellular FA uptake are controversial (35), but transport proteins (FATPs) believed to be involved in FA uptake have been reported (34, 35). Moreover, if melatonin was exerting its inhibitory action via bona fide melatonin receptors (29, 39), these receptors would of necessity be functionally linked to FATP. To determine whether a molecular substrate for melatonin regulation of FA uptake and metabolism was present in hepatoma 7288CTC, we measured the steady-state expression of FATP in tumor and liver tissue using Northern blotting. Northern blot analysis showed that hepatoma 7288CTC overexpressed both the 3.8- and 3.9-kb fragments of FATP (7), whereas liver tissue from these same animals expressed only very low levels of the 2.9 kb fragment (Fig. 6). These data suggest that FATP mRNA expression is linked to progression in hepatoma 7288CTC. An overexpression of the FATP gene favors FA uptake and may protect tumor cells against or allow escape from melatonin's growth-inhibitory influence. Other proteins that are important in nutrient metabolism, energy production, and growth that are not expressed in normal liver may be overexpressed in fast-growing hepatomas (42, 43). To our knowledge, these data are the first to document the expression of FATP mRNA transcripts in a neoplastic tissue. Further studies are aimed at determining whether FATP is involved in facilitated FA

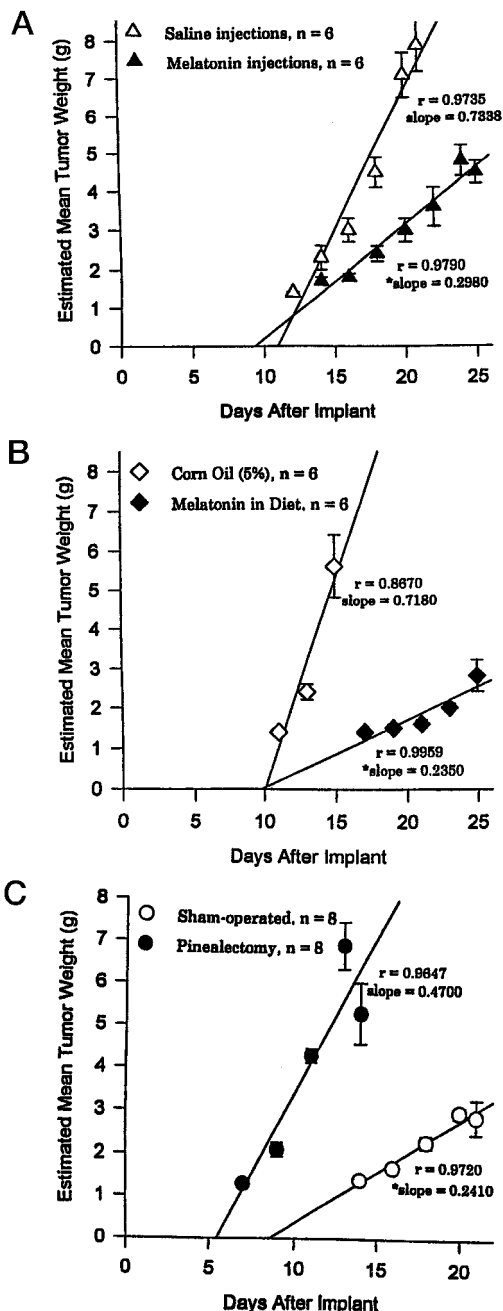


Fig. 5. Effects of melatonin *versus* vehicle injections (A), a melatonin-containing diet *versus* a melatonin-free diet (B), and pinealectomy *versus* sham-pinealectomy (C) on the growth of tissue-isolated hepatoma 7288CTC in adult male Buffalo rats. *Top*, melatonin (200 $\mu\text{g}/0.1$ ml of ethanolic saline) and vehicle injections (0.1 ml of ethanolic saline) were administered s.c. every day in the late afternoon (4:00–6:00 p.m.) to rats provided with a semipurified diet containing 5% corn oil *ad libitum*. *Middle*, rats were fed a semipurified diet containing 5% corn oil containing exogenous melatonin, so that each rat consumed approximately 200 μg of melatonin per day. *Bottom*, rats fed laboratory chow were either pinealectomized or sham-operated. The animals in all treatment groups were maintained on a 12L:12D cycle. Melatonin injections or feeding were initiated 1 week prior to tumor implantation and were continued until the end of the growth experiment. Pinealectomy and sham-pinealectomy procedures were performed 1 week prior to tumor implantation. Regression analyses and tests for parallelism indicated that melatonin treatment (*top* and *middle*) significantly ($*P < 0.05$) decreased the tumor growth rate while pinealectomy (*bottom*) significantly ($*P < 0.05$) increased the rate of tumor growth as compared with corresponding controls.

transport in hepatoma 7288CTC and how its activity is regulated by melatonin. Interestingly, there was no effect of either daily afternoon melatonin injections (200 $\mu\text{g}/\text{day}$) or exposure to constant light on the expression of FATP mRNAs in hepatoma 7288CTC (data not shown)

suggesting no long-term effects of melatonin on FATP gene expression.

Conclusions. On the basis of the results presented here an on pertinent results from the literature (3–11, 16, 22, 29, 38, 39, 44), Fig. 7 depicts a provisional mechanistic model for the signal transduction events that may explain the regulation of LA uptake and metabolism and, thus, the growth of hepatoma 7288CTC in response to low or high circulating melatonin levels *in vivo*. We propose that during the light period (Fig. 7, *top*), when circulating melatonin levels are very low, LA uptake by cancer cells is maximal due to cAMP enhancement of FATP activity, perhaps via the phosphorylation of FATP through a cAMP-dependent protein kinase. Once inside the cell, LA (1–10% of that taken up) is rapidly oxidized to 13-HODE by an EGF-stimulated lipoxygenase that may be associated with the intracellular domain of the EGF receptor. Increased 13-HODE levels then stimulate EGF receptor autophosphorylation and tyrosine phosphorylation of key downstream signal transduction proteins, such as MAPK, resulting in transcriptional activation and enhanced EGF-responsive mitogenesis (10, 11). During the dark period (Fig. 7, *bottom*), when circulating melatonin levels are high, melatonin binds to its cognate receptor(s) (*i.e.*, mt_1 and/or MT_2 ; (Ref. 29), leading to receptor activation and association with an inhibitory G protein. The inhibitory α -subunit of the heterotrimeric G protein then dissociates from the $\beta\gamma$ -subunits and binds to adenylate cyclase, resulting in an inhibition of cAMP production. Decreased cAMP levels may result in diminished FATP activity, possibly due to decreased FATP phosphorylation and a blockade of uptake of LA by cancer cells. As less LA enters cancer cells, less 13-HODE is produced, resulting in suppressed EGF-responsive mitogenesis.

This mechanistic scheme provides a plausible explanation for different tumor growth rates observed during a 12L:12D photoperiod, constant light exposure, or pinealectomy and the administration of exogenous melatonin during a 12L:12D photoperiod. Under diurnal lighting conditions, the melatonin-induced changes in LA uptake and metabolism to 13-HODE fluctuate, as shown in Fig. 1, and the mean tumor growth rate is relatively slow. In pinealectomized or constant light-exposed animals, in which the nocturnal melatonin signal is lost, the balance shifts in favor of stimulation of LA uptake and 13-HODE formation. Thus, EGF-responsive mitogenesis is enhanced throughout the entire 24-h day, culminating in an accelerated latency to tumor palpability and tumor growth rate. Treatment of tumor-bearing rats on a 12L:12D photoperiod with daily, late afternoon melatonin injections or dietary melatonin produces extremely high melatonin levels that summate with the endogenous melatonin signal. This extends the duration of melatonin-suppressed LA uptake, 13-HODE production and EGF-responsive mitogenesis, which slows the latency to tumor palpability and tumor growth rate relative to vehicle-treated controls.

Two long-standing research goals of our laboratory have been to elucidate and understand the mechanisms by which the essential nutrient LA stimulates and the pineal neurohormone melatonin inhibits tumor growth *in vivo*. The bulk of experimental data from both *in vitro* studies indicates that melatonin primarily slows cancer growth promotion/progression by inhibiting the mitogenic actions of a variety of growth factors and hormones, including EFG (45), estradiol (E_2) (23, 24), and prolactin (46). However, the mechanism(s) by which melatonin inhibits tumor growth promotion/progression in the more relevant *in vivo* situation were unknown. These findings that both pharmacological and physiological levels of melatonin block LA uptake and 13-HODE release *in vivo* via a signal transduction pathway involving G protein-coupled melatonin suppression of cAMP provides a potentially unifying mechanism for the first time. Because 13-HODE amplifies the signal transduction pathway responsible for EGF-induced mitogenesis (10, 11), the inhibition of LA uptake and

Table 3 Effects of daily afternoon melatonin injections (200 µg/day), dietary melatonin intake (200 µg/day), constant light exposure, or pinealectomy on mean (± SD) total FA uptake, LA uptake, and 13-HODE release by and FA content of tissue-isolated hepatoma 7288CTC

Tumor FA uptake, content, and 13-HODE release was determined at the end of each of the growth experiments depicted in Fig. 5.

Treatment ^a	Total FA uptake (µg/min/g)	LA uptake (µg/min/g)	Tumor FA content (mg/g)	13-HODE release (ng/min/g)
Saline injected	9.8 ± 0.9	2.0 ± 0.2	32.9 ± 2.6	24.5 ± 1.0
Melatonin injected	5.1 ± 0.5 ^b	1.0 ± 0.1 ^b	13.6 ± 0.4 ^b	1.5 ± 0.3 ^b
Control diet	8.0 ± 1.2	1.4 ± 0.2	31.6 ± 1.0	44.2 ± 9.3
Melatonin-containing diet	2.2 ± 0.6 ^b	0.4 ± 0.01 ^b	7.6 ± 0.5 ^b	ND ^c
Sham-pinealectomized	4.9 ± 0.4	0.9 ± 0.1	15.0 ± 0.2	32.7 ± 2.3
Pinealectomized	7.9 ± 0.8 ^b	1.3 ± 0.2 ^b	114.4 ± 3.5 ^b	209.1 ± 10.0 ^b

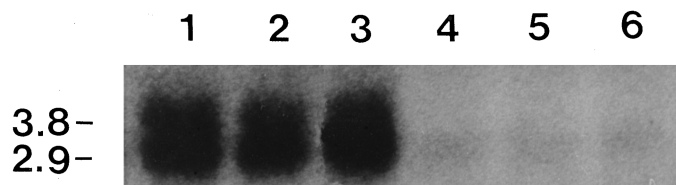
^a There were six to eight animals/tumors per group.^b P < 0.05 vs. respective control.^c ND, not detectable.

Fig. 6. Expression of FATP mRNA in hepatoma 7288CTC. Northern blot analysis showing levels of FATP mRNAs expressed by hepatoma 7288CTC (Lanes 1–3) and liver (Lanes 4–6). Total cellular RNA was extracted from rats maintained for 4 weeks on a 12L:12D cycle. Neither constant light exposure nor treatment with daily afternoon melatonin injections (200 µg) had any effect on expression of FATP mRNA (data not shown).

metabolism to 13-HODE may explain melatonin's capacity to inhibit EGF-induced mitogenesis of MCF-7 human breast cancer cells (45) and SV-40 transformed human fetal retinal pigment epithelial cells (47) *in vitro*. Moreover, the ability of melatonin, in conjunction with EGF, to modulate the transactivation of the estrogen receptor and the activity of MAPK in MCF-7 cells *in vitro* (41) may also be related to its regulation of LA uptake and 13-HODE production.

Interestingly, LA has also been reported to stimulate the proliferation of rat hepatocytes via inhibition of gap junctional intercellular communication (48, 49), whereas melatonin stimulates gap junctional intercellular communication in rat hepatocytes (50) and other cell types (51). As a lipid peroxidation product of LA metabolism (10, 11), 13-HODE, together with other hydroperoxides of LA, may cause free radical damage to biomembranes, particularly connexin molecules involved in gap junctional activity (48). Because melatonin is a potent free-radical scavenger (52, 53), it may protect gap junctions against damage induced by LA-derived, free-radical species, resulting in an enhancement of intercellular communication as an additional mechanism of inhibition of LA-dependent cancer growth.

Here, we describe a novel interface between two seemingly unrelated environmental factors that affect the regulation of tumor growth, namely, dietary fat, as represented by LA, and information about the light/dark cycle, as conveyed by the melatonin signal. The discovery of this interaction forms the basis for a new understanding and integration of widely spread systemic, cellular, and molecular metabolic pathways with the environmental influences of dietary fat, the photoperiod and the circadian system in the maintenance of the host-cancer balance. We believe these results provide a scientific rationale for the development of new dietary recommendations that consider LA intake, circadian-timed melatonin supplementation, and/or photoperiodic alterations for the prevention and treatment of a variety of cancers. Particular consideration may be made for liver cancer, a neoplasm for which there is currently no satisfactory therapy or cure (54).

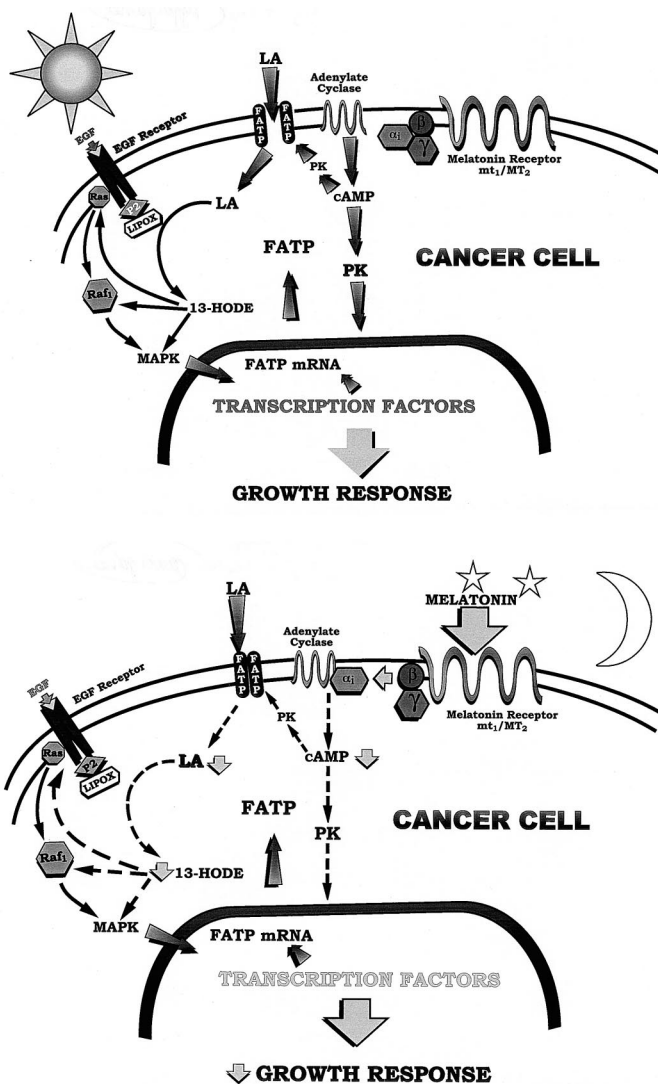


Fig. 7. Provisional model for the signal transduction events that mediate the regulation of LA uptake and metabolism and growth of hepatoma 7288CTC in response to melatonin. (Top), during the light phase (*i.e.*, low melatonin levels), LA uptake by cancer cells is maximal due to elevated levels of cAMP that potentially enhance FATP activity possibly via the phosphorylation of FATP through a cAMP-dependent protein kinase. Intracellular LA is oxidized to 13-HODE by an EGF-stimulated lipoxygenase that may be associated with the EGF receptor through an adapter protein (protein-2) (10, 11). Increased 13-HODE levels stimulate EGF receptor autophosphorylation and tyrosine phosphorylation of key downstream signal transduction proteins such as Ras, Raf₁, and MAPK resulting in transcriptional activation and enhanced EGF-responsive mitogenesis (10, 11). (Bottom), during the dark phase (*i.e.*, high melatonin levels), melatonin binds to its receptor(s) mt₁ and/or MT₂, leading to receptor activation and association with an inhibitory G protein. The inhibitory α -subunit dissociates and binds to adenylate cyclase resulting in an inhibition of cAMP production. Decreased cAMP levels result in decreased activity of FATP and, thus, decreased LA uptake and 13-HODE production, leading to diminished EGF-responsive mitogenesis.

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