Comparative Medicine
Copyright 2003
by the American Association for Laboratory Animal Science

Vol 53, No 2
April 2003
Pages 186-190

Physiologic Melatonin Concentration, Omega-3 Fatty Acids, and Conjugated Linoleic Acid Inhibit Fatty Acid Transport in Rodent Hind Limb Skeletal Muscle In Vivo

Robert T. Dauchy, David E. Blask, PhD, MD,* Leonard A. Sauer, MD, PhD, Leslie K. Davidson, Jean A. Krause, Laura C. Smith, and Erin M. Dauchy

Melatonin (MLT), the circadian neurohormone secreted by the pineal gland in mammals during darkness, eicosapentanoic acid (EPA), and conjugated linoleic acid (CLA) have established regulatory roles in cancer growth. Investigations in our laboratory have indicated that these agents inhibit fatty acid (FA) transport by tumors and several sub-types of white adipose tissue via inhibitory G protein-coupled receptor mechanisms. Skeletal muscle constitutes over 45% of human body mass and plays an important role in cancer cachexia and obesity-related diseases. Since fatty acid oxidation is a major source of energy for this tissue, we tested the hypothesis that physiologic MLT levels, EPA, or CLA injected intravenously, inhibit FA uptake in rat skeletal muscle in vivo. We used a surgical technique for catheterizing the femoral vein in rats that allows rapid blood collection from the entire hind limb, while ensuring continuous blood flow to the tissue. Blood acid/gas tensions and hematocrit were monitored and remained constant during the course of each experiment. The MLT, EPA, and CLA inhibited FA uptake by the tissue and lowered cAMP values. Glucose uptake and glycerol production in the hind limb were not affected. These investigations suggest a novel role for MLT, omega-3 FAs, and CLA in the regulation of FA transport and fat metabolism in skeletal muscle.

Increased dietary intake of total fatty acids (TFAs), and more specifically, linoleic acid (LA), promotes carcinogenesis, obesity, and diabetes (1). Conversely, in experimental animals, these disease states appear to be ameliorated by physiologic concentration of the circadian neurohormone melatonin (MLT) that is produced by the pineal gland during the night (2, 3), as well as omega-3 (ω-3) fatty acids and conjugated LA (CLA; 10t,12c isomer) (4, 5). Muscle tissue, comprising over 45% of the body weight of adult animals (6), responds rapidly to diet, nutritional stress, and metabolic and hormonal influences. Fatty acid oxidation is a major source of energy for skeletal muscle tissue and is directly associated with these and other metabolic processes (7). This suggests that inhibition of TFA uptake by skeletal muscle tissue may have an impact on the development and progression of a variety of disease states.

Results of recent studies in our laboratory (4, 5, 8) indicated that physiologic concentration of MLT (10^{-9} *M*, nighttime blood value), as well as the ω-3 fatty acid EPA (0.5 m*M*), markedly inhibit uptake of TFA and LA by rodent hepatomas and human cancer xenografts; in addition, CLA (10t, 12c isomer, 0.1 m*M*) suppresses TFA/LA uptake by rodent hepatomas. Inhibition occurs via a G protein-coupled receptor-mediated decrease in cAMP values in the tumor tissue, which in turn suppresses tumor growth and metabolism. The objective of the study reported here was to test the effects of several hormonal and dietary agents on TFA uptake by rodent skeletal muscle tissue in the healthy, fed rat. In addition, glucose metabolism, glycerol production, and cAMP and protein concentrations were measured. The surgical catheterization procedure used here allows continuous and unimpeded blood flow to and from the entire rodent hind limb, using host whole blood.

The in vivo procedure described here is a modification of the original technique developed in this laboratory that was designed for whole blood perfusion of "tissue-isolated" tumors (10). To our knowledge, this technique has not been used before. The system described here also provides continuous flow to the entire rat hind limb with normal host blood, thus reducing the risks of ischemia and alterations in tissue metabolic and physiologic parameters, such as O_2 uptake/CO_2 production, blood pH, hematocrit, and substrate utilization/productions rates. Some investigators made use of recirculating perfusion systems, using prepared, non-erythrocyte buffer systems (11, 12), or flow-through systems (13, 14) containing erythrocytes and albumin, whereas others (15) used flow-through systems with erythrocyte/albumin-free perfusates. Difficulties encountered using many of these perfusion systems include cessation of blood flow to the tissue and resultant ischemia, accumulation of metabolic by-products, and decrease in substrate concentration, well-known physiologic and metabolic complications associated with artificially prepared perfusion media (15-17).

Aberrations of lipid metabolism during cancer promotion, progression, cancer cachexia, and obesity-related diseases, are

Received: 10/04/02. Revision requested: 1/03/03. Accepted: 1/21/03.
Laboratory of Experimental Neuroendocrinology/Oncology, Bassett Research Institute, Cooperstown, New York 13326-1394.
**Corresponding author.*

directly associated with skeletal muscle tissue metabolism and physiology (18). As such, and, in view of the importance of lipid metabolism to skeletal muscle tissue function, a study of the influence of MLT and dietary agents EPA and CLA on rat hind limb skeletal muscle was of interest. The in vivo model presented here provides a convenient system to examine the effects of these agents on muscle metabolism in the laboratory rat.

Materials and Methods

Animals, housing conditions, and diet. Adult male (250 to 300 g) specific-pathogen-free Sprague Dawley rats (Hsd:Sprague Dawley [SD]) were obtained from Harlan (Indianapolis, Ind.). All animals were maintained in environmentally controlled rooms (23°C; 45 to 50% humidity) in isolator units (Thoren Caging Systems, Hazelton, Pa.) in a facility that has animal care and use programs accredited by AAALAC International and in accordance with the *Guide for the Care and Use of Laboratory Animals*. This project was approved by the Institutional Animal Care and Use Committee. On arrival and during the course of this study, animals experienced 12:12-h light:dark (lights on 6 a.m. to 6 p.m.; 300 lux). The animal facility was completely light tight; no lights from external sources entered the room during the dark phase. During the course of this study, serum samples from sentinel rats were tested by ELISA to ensure that the animals were not infected with bacterial or viral agents (Comprehensive Health Monitoring Program: Charles River Laboratories, Kingston, N.Y.). Animals were given ad libitum access to water and an essential fatty acid-replete diet (Prolab rat, mouse, and hamster 1000 formula, Agway, Syracuse, N.Y.). Quadruplicate determinations of six separate batches indicated that this diet contained 4.1 g of TFA/100 g of diet composed of 1.3% myristic (C14:0), 22.7% palmitic (C16:0), 2.6% palmitoleic (C16:1n7), 12.3% stearic (C18:0), 33.1% oleic (C18:1n9), 25.7% linoleic (C18:2n6), and 0.2% arachidonic (C20:4n6) acids. Minor amounts of other FA comprised 2.1%. More than 93% of the TFA content was in the form of triglycerides and phospholipids; more than six percent was present as free fatty acids (FFA). Food was not withheld from animals before each experiment, and the skeletal muscle of the hind limbs was in a resting state.

Surgical procedures. Animals were anesthetized by intraperitoneal administration of sodium pentobarbital (25 mg/kg of body weight; Abbott Laboratories, North Chicago, Ill.) into the lower right quadrant. During these procedures, animals breathed air unassisted and were maintained in the supine position on a heating pad; body core temperature was maintained at 37°C. The right carotid artery was catheterized with PE-50 tubing (Clay Adams, Parsippany, N.J.) attached to a one-milliliter tuberculin syringe (Becton Dickinson and Co., Franklin Lakes, N.J.) containing sodium heparin (50 U/100 g of body weight; Elkins-Sinn, Inc., Cherry Hill, N.J.).

The surgical procedure used here is a modification of the original work of Ruderman and co-workers (7) and Dauchy and co-workers (10). However, this technique allows continuous blood flow to the hind limb at all times, thus preventing the damaging effects of ischemia. The left femoral artery and vein were exposed via a three-centimeter incision in the inguinal fossa region. Ligatures were placed around the femoral vein. A butterfly ST infusion catheter (25-gauge × 3/8-in.; Abbott Laboratories, North Chicago, Ill.) was inserted into the femoral vein, followed by application of a tight ligature to hold the catheter in place; venous blood flowed passively from all major muscle groups of the hind limb. Initial arterial (carotid) and venous (femoral) blood samples were collected (0.5 ml) in tubes chilled on ice. One minute prior to the end of this control blood collection, a specified amount of melatonin (0.15 ml of $10^{-7}M$; final blood concentration, $10^{-9}M$); EPA (0.15 ml of $5 \times 10^{-2}M$; final blood concentration, 0.5 m*M*); CLA (10t,12c)—0.15 ml of $10^{-2}M$ with final blood concentration of 0.1 m*M*; arachidonic acid (0.15 ml of $10^{-1}M$ with final blood concentration of 1.31 m*M* [control to EPA]); or CLA (9c,11t)—0.27 ml of $10^{-2}M$ to a final concentration of 0.18 m*M* (control to CLA [10t,12c]), prepared in plasma from blood prior to treatment, was infused into the right external jugular vein (sample injections based on an estimated animal whole blood volume of 15 ml). One additional arterial and a venous blood sample (0.5 ml) were collected. Separate samples were collected simultaneously for hematocrit, pH, and blood gas analyses. The MLT, EPA, and arachidonic acid were obtained from Sigma Scientific (St. Louis, Mo.); CLA isomers were obtained from Cayman Chemicals (Ann Arbor, Mich.).

Lipid extraction and analysis. Following each cannulation procedure, the muscles of the hind limb (soleus, plantaris, a portion of the red gastrocnemius from the deep medial head, biceps, and tibialis anterior) were rapidly freeze-clamped with metal tongs pre-cooled under liquid nitrogen, weighed, then stored at –80°C. Arterial and hind limb venous blood TFA were extracted from plasma by use of the method of Folch and co-workers (19), as modified by McDonald-Gibson (20). Heptadecanoic acid was added as an internal standard, and analyses were carried out in duplicate. Methyl esters of FA were analyzed, using a Hewlett Packard (Palo Alto, Calif.) model 5890A Gas Chromatograph equipped with a flame ionization detector (model 7673 A), autoinjector (model 7673S), and integrator (model 3396A). Separations were carried out by use of a 0.25-mm × 30-m capillary column (model 2380; Supelco, Inc., Bellefonte, Pa.) at 190°C, with helium as the carrier gas (linear rate, 20 cm/s; split 100:1). The injection port and detector were set at 220°C. All FA methyl esters were identified on the basis of their retention time, compared with that of known standards.

Determination of 13(S)-hydroxy-9Z,11E-octadecadienoic aid (13-HODE). Plasma samples were prepared for high-performance liquid chromatography analysis of 13-HODE, using a modification of the method of Glasgow and co-workers (21). The samples were extracted and analyzed as described (9).

Whole blood glucose and glycerol determinations. Arterial and venous plasma samples were analyzed for glucose by use of the Roche Diagnostics kit (Roche Diagnostics, Roswell, Ga.) and for glycerol content by use of the Triglyceride GPO-Trinder kit (No. 337-B, Sigma Scientific) with the Roche Cobas Mira *plus* spectrophotometer (Branchburg, N.J.).

Tumor protein determinations, cAMP analysis. Tissue homogenates (10%) were prepared from the frozen samples, and total protein determinations were carried out, using Coumassie blue stain; results were cross-checked, using the biuret method (22) as adapted for the Roche Cobas Mira *plus* spectrophotometer. Measurement of tissue cAMP concentrations was carried out, using the Amersham Pharmacia Biotech Biotrak enzyme immunoassay (EIA) system (RPN 225; Piscataway, N.J.).

Statistical analysis. Results were expressed as the mean ± one standard deviation, six animals per group; we used one-way analysis of variance (ANOVA) followed by the Student-Newmann-Keuls

Table 1. Skeletal muscle total fatty acids (TFA) uptake, glucose uptake, and glycerol production

Group (n = 6/group)	TFA uptake (µg/min/g)		Glucose uptake (µg/min/g)		Glycerol production (µg/min/g)	
	Fed	Treatment	Fed	Treatment	Fed	Treatment
Fed control	5.60 ± 1.26	...	6.67 ± 1.36	...	0.129 ± 0.057	...
Fed >> MLT	7.25 ± 1.47[a]	(1.20) + 0.55[b]	13.76 ± 4.06	13.27 ± 2.10	0.157 ± 0.055	0.180 ± 0.099
Fed >> CLA (10t, 12c)	5.20 ± 1.58[a]	(0.39) + 0.6[b]	6.43 ± 2.94	6.58 ± 2.67	0.432 ± 0.215	0.414 ± 0.237
Fed >> EPA	5.74 ± 1.12[a]	(0.05) + 0.98[b]	5.17 ± 2.73	5.13 ± 2.47	0.469 ± 0.383	0.502 ± 0.375
Fed >> Arach	6.61 ± 0.35[a]	6.89 + 1.14	13.04 ± 5.3	10.72 ± 4.47	0.157 ± 0.144	0.123 ± 0.138
Fed >> CLA (9c, 11t)	6.29 ± 3.10[a]	6.90 + 1.70	10.55 ± 3.40	8.47 ± 4.80	0.120 ± 0.111	0.180 ± 0.074

[a] vs. [b]Significant difference, $P < 0.05$.
(Value) indicates TFA release by muscle.
MLT = Melatonin; CLA = conjugated linoleic acid; EPA = eicosapentanoic acid;and Arach = arachidonic acid.

multiple comparison test to evaluate differences. Mean differences among groups were considered statistically significant at $P < 0.05$.

Results

Whole blood acid/gas analysis. Whole blood acid/gas analysis for experiments in each of the study groups (n = 6/group), arterial and venous hematocrits, and venous flow rate data were not significantly different between treatment and control perfusions. Compared with control values, those for arterial and venous hematocrit did not differ significantly from each other and with those after treatment, revealing only minor differences due to hemocentration (< 1%) by the tissue. As a consequence, the data presented here represent the combined values for the control and treatment measurements. Arterial and venous hematocrits measured 47.4 ± 2.2 and 48.2 ± 2.2%, respectively (n = 36). Arterial/venous measurements for blood pH, pO_2, and pCO_2 were 7.41 ± 0.02/7.29 ± 0.03, 160.2 ± 8.9/34.2 ± 5.7 mmHg, and 29.5 ± 7.0/60.8 ± 5.3 mmHg (n = 36), respectively. Venous flow rate (0.278 ± 0.11 ml/min, n = 36) did not fluctuate during the course of each collection nor did it vary significantly between pre-treatment and treatment regimens during the course of each five-minute experiment.

Skeletal muscle TFA and glucose uptake, and glycerol production. The surgical technique described previously was designed to simulate actual in vivo conditions. Animals were provided ad libitum to food and water, constant environmental living conditions within the animal facility, and surgical regimens that did not vary. Experiments were conducted between the hours of 7 and 9 a.m. daily. Mean uptake of TFA and glucose, as well as glycerol production by the muscle tissue is presented in Table 1. In all experiments, resting, pre-treatment values for TFA uptake were not significantly different from those for the non-treatment, resting, control muscle samples. These TFA uptake rates correlate well with arterial TFA supply rates to and venous effluent rates from skeletal muscle tissue: 38.50 ± 3.31 and 32.38 ± 3.24 µg/min/g (n = 30), respectively, which represented an uptake of 16.0 ± 1.8% of arterial TFA supply to the tissue. The TFA uptake values following treatment with either MLT, CLA (10t, 12c), or EPA, compared with fed control values, indicated a clear inhibitory effect. Following these treatments, TFA uptake as a percentage of arterial TFA supply was –1.8 ± 1.1%, –0.4 ±1.1%, and –0.2 ± 0.4%, respectively; compared with control values, these values were significantly ($P < 0.05$) different. It should be noted here that, although CLA (10t, 12c) and EPA caused this substantial inhibition of TFA by the skeletal muscle, these agents were themselves taken up by the tissue. These experiments indicated uptake of 0.52 ± 0.21 and 2.11 ± 1.12 µg/min/g of CLA (10t, 12c) and EPA, respectively, by the tissue; this represented 44.5 ± 7.1 and 51.5 ± 13.8% uptake of CLA (10t,12c) and EPA, respectively, by the hind limb, as a percentage of arterial supply. Treatment with arachidonic acid [C20:4(5,8,11,14)], an ω-6 fatty acid (control FA) of similar size and structure to that of ω-3 EPA [C20:5(5,8,11,14,17)] but with one fewer double-bond, did not have an inhibitory affect on TFA uptake. Likewise, in experiments with CLA (9c, 11t), another isomer of CLA, there was no inhibitory effect on TFA uptake.

Table 2. Skeletal muscle cAMP and tissue protein concentrations

Group (n = 6/group)	cAMP (nmol/g of tissue)	Protein (mg/g)
Fed controls	0.518 ± 0.113[a]	268.9 ± 14.6
Fed >> MLT	0.166 ± 0.060[b]	265.0 ± 26.9
Fed >> CLA (10t,12c)	0.201 ± 0.009[b]	248.0 ± 48.5
Fed >> EPA	0.169 ± 0.004[b]	250.6 ± 27.1
Fed >> Arach	0.447 ± 0.137	250.0 ± 20.8
Fed >> CLA (9c, 11T)	0.323 ± 0.029	248.3 ± 19.7

[a] vs. [b]Significant difference, $P < 0.05$.
See Table 1 for key.

Interestingly, there was no detectable production of 13-HODE, the mitogenically active metabolite of LA in tumor cells (9), by skeletal muscle tissue in any of the experiments. Correspondingly, glucose uptake and glycerol production rates were unchanged prior to and following treatment by any of the experimental substances. Mean glucose uptake and glycerol production by the entire resting hind limb was 9.07 ± 3.32 and 0.260 ± 0.156 µg/min/g (n = 30), respectively. Glucose uptake by the muscle tissue represented approximately 21.9 ± 6.8% of the arterial supply, whereas glycerol production rate reflected a 27.4 ± 9.8% increase over arterial values.

Tissue cAMP and protein analyses. Homogenates of frozen skeletal muscle tissue specimens were extracted and analyzed for cAMP and total protein content (Coomasie blue method). Results of these analyses are presented in Table 2. Compared with values for fed controls, there were significantly ($P < 0.05$) lower amounts of cAMP in MLT-, CLA (10t, 12C)-, or EPA-treated groups. However, there were no significant differences between the arachidonic acid or CLA (9c, 11t) treatment groups, compared with the controls. Analysis of total tissue protein did not reveal significant differences between control and treatment groups.

Discussion

In previous studies of rat hepatomas 7288CTC and 7777 (23, 24), and subsequently, human breast cancer xenografts (25), we reported that tumor growth and metabolism were dependent on LA concentrations and tumor uptake of this essential FA. As the dietary concentration and animal intake of LA increases, plasma

values are concomitantly increased. Rates of tumor growth are maximal because higher cAMP values enhance FA transport, thus allowing increased amounts of LA to enter the tumor cell. Within the tumor cell, the enzyme, 15-lipoxygenase, converts LA to a potent mitogenically active metabolite, 13-HODE, which enhances stimulation of tumor growth by the EGFR/MAPK pathway. Further studies indicated that the circadian neurohormone MLT, ω-3 FAs, and an isomer of conjugated linoleic acid, CLA (10t, 12c) markedly inhibited the uptake of LA by these tumor tissues (8, 25, 26). This occurs via a receptor-mediated G_{I-} inhibitory protein that suppresses adenylyl cylase activation, lowering of cAMP concentration. More recently, it was documented that a similar effect also takes place in the white adipose tissue of the inguinal fat depot of rats (26, 27).

The study reported here was designed to determine whether any of these phenomena occurred in rat hind limb skeletal muscle. Since lipid metabolism in skeletal muscle, much like that in white adipose tissue, is affected by cancer, cachexia, and obesity-related diseases, the skeletal muscle of the rat hind limb proved to be an important candidate for investigation. In the normal physiologic state, energy production in skeletal muscle tissue is derived from the oxidative metabolism of glucose and FAs (28), which in turn, can influence concentrations of ATP, ADP, and cAMP in the tissue. Under the changing metabolic conditions in cancer progression, cachexia, or obesity, the oxidative metabolism of one substrate may predominate over another. Disruptions in normal circadian hormone concentrations (29) or dietary imbalances (9, 26) may well induce and promote progression of these disease states. The controlling factors for the physiologic alterations involved with these aberrant states are still unclear.

An important advantage of this model system was the ability to carry out rapid blood collection from single passage through the tissue, with little or no alteration in blood flow, pH and gas tensions. Since the actual surgical procedure caused minimal disturbance to the tissue, and rat blood hemoglobin was the natural oxygen carrier, blood flow rate and physiologic values of all nutrients were present and maintained at all times. As a result, the minor surgical manipulation used here had little or no effect on substrate uptake and metabolism by the tissue.

It has been determined that, with aging, plasma MLT concentration decreases markedly in all vertebrate species, including rats (30); this decrease is associated with increasing concentrations of visceral fat, plasma leptin and insulin (31) in the animal, all of which affect directly and indirectly skeletal muscle physiology and metabolism (32). Increased consumption of LA-enriched diets increase tumorigenesis and the growth of transplanted tumors, including human xenografts (25, 33). On the other hand, consumption of diets rich in ω-3 FAs, including α-linolenic (C18:3n3), EPA, or docosahexaenoic acid (C22:6n3) inhibit tumorigenesis and growth (34). One consideration may be the overall availability of substrate to the tissue, and overall net energy contribution for the tissue and host. In the study reported here, the muscle of the healthy, nontumor-bearing, nonobese animal experienced little or no changes in glucose uptake, despite the marked inhibitory effect of MLT, CLA (10t, 12c), or EPA on lipid uptake and cAMP production. The inhibition of cAMP and muscle TFA uptake by a physiologic concentration of MLT strongly indicates that this effect is mediated by an inhibitory G protein-coupled MLT receptor mechanism. We have observed such a mechanism to be operational in MLT-inhibited uptake of TFA by tumors and adipose tissue in vivo (8, 26).

Reimer and co-workers (32) were the first investigators to determine that, in the perfused rat hind limb, 75% of TFA was incorporated into muscle lipids, 10% was oxidized to CO_2, and 5% was recovered in bone lipids; more than 70% of the FAs incorporated were found in the triglyceride fraction. Those investigators also reported that TFA concentrations and uptake were independent and unassociated with either glucose uptake or glycerol production by the skeletal muscle tissue, in agreement with our findings in vivo using host blood. This suggests that the inhibitory effect of MLT, CLA (10t, 12t), and EPA on TFA uptake is not controlled by either arterial TFA concentration or glucose uptake. With respect to tumor tissue (25), inguinal fat pads (26, 27), and skeletal muscle, current evidence indicates that the uptake of ω-3 FAs and CLA occurs independently of uptake of other FAs. Although uptake of other saturated, monounsaturated, and ω-6 polyunsaturated FAs was completely suppressed by either EPA or CLA, uptake of these inhibitory FAs continued.

The underlying mechanism behind this observation is not clearly understood. The traditional school of thought supports cell membrane diffusion by FAs, which occurs as a result of their lipophilic nature (35). A growing body of more recent evidence, however, suggests that transport of FAs occurs via a specific carrier-mediated pathway (36). Indeed, the Fatty Acid Transport 1 gene (*FATP1)* is highly expressed in tissues with high rates of metabolism such as the heart, adipose tissue, testis, intestine, hepatoma, and skeletal muscle (37-39), and in tumor tissues, expression may be linked to cancer progression (40). It should be pointed out that the possible effects of CLA (10t, 12c) on skeletal muscle tissue are not clearly understood. The evidence suggests that lean muscle mass increases relative to fat gain; this is due to enhanced beta-oxidation of FA, while TFA uptake in white adipose tissue is inhibited, particularly in younger rats (41). To our knowledge, this may be the first evidence of a direct blockade of TFA uptake in skeletal muscle in vivo by the CLA (10t, 12c) isomer.

Here we described a unique interaction between two environmental factors that regulate metabolism in skeletal muscle. The circadian neurohormone MLT, and dietary fats, including the ω-3 FA, EPA, and CLA (10t, 12c), play a major regulatory role in overall TFA uptake and metabolism in this important tissue. Investigations now underway, using an adaptation of the surgical technique presented here, will allow rapid perfusion of the rat hind limb skeletal muscle. In future studies, we will test for the presence of the melatonin receptors (MT1 and MT2) in an attempt to more clearly define the molecular and metabolic pathways associated with this important tissue and the host. We believe the results presented here, using this novel surgical model system, help provide a better understanding for the role of lipid metabolism in cancer, cachexia, and obesity-related diseases.

Acknowledgments

We thank Darin Lynch for his expert care of the laboratory animals and assistance in preparation of tissue homogenates. This work was supported in part by grant number RO1CA76197 from the National Cancer Institute (DEB) and the Stephen C. Clark Research Fund (DEB).

References

1. **Committee on Diet, Nutrition and Cancer—National Research Council.** 1982. 73-105. National Academy Press, Washington, D.C.
2. **Reiter, R. J.** 1991. Pineal melatonin: cell biology of its synthesis and of its physiological interactions. Endocrin. Rev. **12:**151-180.
3. **Pierrefiche, G., G. Topall, G. Courbain, I. Henrier, and H. Laborit.** 1993. Antioxidant activity of melatonin in mice. Res. Commun. Chem. Pathol. Pharmacol. **80(2):**211-223.
4. **Sauer, L. A., R. T. Dauchy, and D. E. Blask.** 2000. Mechanism for the antitumor and anticachetic effects of n-3 fatty acids. Cancer Res. **60:**5289-5295.
5. **Sauer, L. A., R. T. Dauchy, D. E. Blask.** 2002. Conjugated linoleic acid (CLA) inhibits fatty acid (FA) uptake and growth in hepatoma 7288CTC and FA transport in inguinal fat pads in Buffalo rats via a G_i protein-coupled signal transduction pathway. Fed. Am. Soc. Exp. Biol. **16(5):** Abst. 733, p. A997.
6. **Wannamacher, R. W.** 1975. Protein metabolism (applied biochemistry), p. 85-153. *In* J. Ghadimi (ed), Total parental nutrition, premises, and promises, Wiley and Sons Inc., New York.
7. **Ruderman, N. B., C. R. S. Houghton, and R. Hems.** 1971. Evaluation of the isolated perfused rat hindquarter for the study of muscle metabolism. Biochem J. **124:**639-651.
8. **Blask, D. E., L. A. Sauer, R. T. Dauchy, E. W. Holowachuk, M. S. Ruhoff, and H. S. Kopff.** 1999. Melatonin inhibition of cancer growth in vivo involves suppression of tumor fatty acid metabolism via melatonin receptor-mediated signal transduction events. Cancer Res. **59:**4693-4701.
9. **Sauer, L. A., R. T. Dauchy, D. E. Blask, B. J. Armstrong, and S. Scalici.** 1999. 13-Hydroxyoctadecadienoic acid is the mitogenic signal for linoleic acid-dependent growth in rat hepatoma 7288CTC in vivo. Cancer Res. **59:**4688-4692.
10. **Dauchy, R. T. and L. A. Sauer.** 1986. Preparation of "tissue-isolated" rat tumors for perfusion: a new surgical technique that preserves a continuous blood Flow. Lab. Anim. Sci. **36(6):**678-681.
11. **Shiota, M., S. Golden, and J. Katz.** 1984. Lactate-metabolism in the perfused rat hindlimb. Biochem. J. **222:**281-292.
12. **Rennie, M. J. and J. Holloszy.** 1977. Inhibition of glucose uptake and glycogenolysis by availability of oleate in well-oxygenated perfused skeletal muscle. Biochem. J. **168:**737-743.
13. **Chiasson, J.-L. H. Shikama, D. T. W. Chu, and J. H. Exton.** 1981. Inhibitory effect of epinephrine on insulin-stimulated glucose uptake by rat skeletal muscle. J. Clin. Invest. **68:**706-713.
14. **Masakazu, S. and T. Sugano.** 1986. Characteristics of rat hindlimb perfused with erythrocyte- and albumin-free medium. J. Am. Physiol. Soc. **251:**C78-C84.
15. **Lewis, S. B., T. A. Schultz, D. K. Westbie, J. E. Gerich, and J. D. Wallen.** 1977. Insulin-glucose dynamics during flow-through perfusion of the isolated rat hindlimb. Hormone Metab. Res. **9:**190-195.
16. **Preedy, V. R., V. M. Pain, and P. J. Garlick.** 1984. The metabolic state of muscle in the isolated perfused rat hemicorpus in relation to rates of protein synthesis. Biochem. J. **218:**429-440.
17. **Berger, M., L. A. Haag, M. Goodman, and N. B. Ruderman.** 1976. Glucose metabolism in perfused skeletal muscle. Biochem. J. **158:**191-202.
18. **Tisdale, M. J.** 1993. Cancer cachexia. Anticancer Drugs **4:**115-125.
19. **Folch, J. M., M. Lees, and G. H. Sloane-Stanley.** 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. **226:**497-507.
20. **McDonald-Gibson, R. G.** 1987. Quantitative measurements of arachidonic acid in tissues or fluids, p. 259-268. *In* C. Benedetto, R. G. McDonald-Gibson, S. Nigam, T. F. Slater (ed.), Prostaglandins and related substances. IRL Press, Washington, D.C.
21. **Glasgow, W. C., r. Hui, A. L. Everhart, S. P. Jayawickreme, J. Angerman-Stewart, B-B. Han, and T. E. Eling.** 1997. The linoleic acid metabolite, (13S)-hydroperoxyoctadecadienoic acid, augments the epidermal growth factor receptor signaling pathway by attenuation of receptor dephosphorylation. Differential response in Syrian hamster embryo tumor suppressor phenotypes. J. Biol. Chem. **272:**19269-19276.
22. **Szarkowska, L. and M. Klingenberg.** 1963. The biuret method for protein determination. Biochem. J. **338:**674.
23. **Sauer, L. A., R. T. Dauchy, and D. E. Blask.** 1997. Dietary linoleic acid intake controls the arterial blood plasma concentration and rates of growth and linoleic acid uptake and metabolism in hepatoma 7288CTC in Buffalo rats. J. Nutr. **127:** 1412-1421.
24. **Sauer, L. A. and R. T. Dauchy.** 1992. Uptake of plasma lipids by tissue-isolated hepatomas 7288CTC and 7777 in vivo. Br. J. Cancer **66:**290-296.
25. **Sauer, L. A., D. E. Blask, and R. T. Dauchy.** 2001. Mechanism of the antitumor effect of n-3 fatty acids and MCF-7 human breast cancer xenografts perfused in situ in nude rats. Am. Assoc. Cancer Res. **42:**Abst.1680, p. 311.
26. **Sauer, L. A., R. T. Dauchy, and D. E. Blask.** 2001. Melatonin inhibits fatty acid transport in inguinal fat pads of hepatoma 7288CTC-bearing and normal Buffalo rats via receptor-mediated signal transduction. Life Sci. **68:**2835-2844.
27. **Dauchy, R. T., D. E. Blask, and L. A. Sauer.** 2000. Preparation of the inguinal fat pad for perfusion in situ: a surgical technique that preserves continuous blood flow. Contemp. Top. Lab. Anim. Sci. **39:**29-33.
28. **Wolfe, R. R.** 1998. Metabolic interactions between glucose and fatty acids in humans. Am. J. Clin. Nutr. **67:**519-526S.
29. **Blask, D. E., L. A. Sauer, and R. T. Dauchy.** 2002. Melatonin as a chronobiotic/anticancer agent: cellular, biochemical, and molecular mechanisms of action and their implications for circadian-based cancer therapy. Curr. Topics Med. Chem. **2:**113-132.
30. **Pang, S. F., C. W. Tsang, G. X. Hong, P. C. Vip, P. L. Tang, and G. M. Brown.** 1990. Fluctuation of blood melatonin concentrations with age: result of changes in pineal melatonin secretion, body growth and aging. J. Pineal Res. **8:** 179-192.
31. **Rasmussen, D. D., B. M. Boldt, C. W. Wilkinson, S. M. Yellon, and A. M. Matsumoto.** 1999. Daily melatonin administration at middle age suppresses male visceral fat, plasma leptin, and plasma insulin to youthful levels. Endocrinology **140(2):**1009-1012.
32. **Reimer, F., G. Loffler, G. Hennig, and O. H. Wieland.** 1975. The influence of insulin on glucose and fatty acid metabolism in the isolated perfused rat hind quarter. Z. Physiol. Chem. **356(S):**1055-1066.
33. **Reddy, B. S., C. Burill, and J. Rigotty.** 1991. Effects of diets high in omega-3 and omega-6 fatty acids on initiation and postinitiation stages of colon carcinogenesis. Cancer Res. **51:**487-491.
34. **Rose, D. A. and J. M. Connolly.** 1993. Effects of dietary omega-3 fatty acids on human breast cancer growth and metastasis in nude mice. J. Natl. Cancer Inst. **85:**1743-1747.
35. **Zakim, D.** 1996. Fatty acids enter cells by simple diffusion. Proc. Soc. Exp. Biol. Med. **212:**5-14.
36. **Fitscher, B. A., C. Elsing, H-D. Riedel, J. Gorski, and W. Strummel.** 1996. Protein-mediated facilitated uptake processes for fatty acids, bilirubin, and other amphipathic compounds. Proc. Soc. Exp. Biol. Med. **212:**15-23.
37. **Schaffer, J. E. and H. F. Lodish.** 1994. Expression cloning and characterization of a novel adipocyte long chain fatty acid transport protein. Cell **79:**427-436.
38. **Hirsch, D., A. Stahl, and H. F. Lodish.** 1998. A family of fatty acid transporters conserved from *Mycobacterium* to man. Proc. Natl. Acad. Sci. USA **95:**8625-8629.
39. **Fronhert, B. I., T. Y. Hui, and D. A. Berhlohr.** 1999. Identification of a functional peroxisome proliferator-response element in the murine fatty acid transport protein gene. J. Biol. Chem. **274:**3970-3977.
40. **Blask, D. E., R. T. Dauchy, L. A. Sauer, and J. A. Krause.** 2002. Dietary melatonin inhibits tumor growth via melatonin receptor-mediated suppression of tumor linoleic acid (LA) uptake and metabolism. Am. Assoc. Cancer Res. **43:** Abst. 3203, p. 228.
41. **Pariza, M. W., Y. Park, and M. E. Cook.** 2001. The biologically active isomers of conjugated linoleic acid. Prog. Lipid Res. **40:**283-298.