

# Triglycerides Induce Leptin Resistance at the Blood-Brain Barrier

William A. Banks,<sup>1</sup> Alan B. Coon,<sup>1</sup> Sandra M. Robinson,<sup>1</sup> Asif Moinuddin,<sup>1</sup> Jessica M. Shultz,<sup>1</sup> Ryota Nakaoka,<sup>1,2</sup> and John E. Morley<sup>1</sup>

**Obesity is associated with leptin resistance as evidenced by hyperleptinemia. Resistance arises from impaired leptin transport across the blood-brain barrier (BBB), defects in leptin receptor signaling, and blockades in downstream neuronal circuitries. The mediator of this resistance is unknown. Here, we show that milk, for which fats are 98% triglycerides, immediately inhibited leptin transport as assessed with in vivo, in vitro, and in situ models of the BBB. Fat-free milk and intralipid, a source of vegetable triglycerides, were without effect. Both starvation and diet-induced obesity elevated triglycerides and decreased the transport of leptin across the BBB, whereas short-term fasting decreased triglycerides and increased transport. Three of four triglycerides tested intravenously inhibited transport of leptin across the BBB, but their free fatty acid constituents were without effect. Treatment with gemfibrozil, a drug that specifically reduces triglyceride levels, reversed both hypertriglyceridemia and impaired leptin transport. We conclude that triglycerides are an important cause of leptin resistance as mediated by impaired transport across the BBB and suggest that triglyceride-mediated leptin resistance may have evolved as an anti-anorectic mechanism during starvation. Decreasing triglycerides may potentiate the anorectic effect of leptin by enhancing leptin transport across the BBB. *Diabetes* 53:1253–1260, 2004**

**L**eptin is a 16-kDa protein secreted by fat cells (1) that regulates feeding and energy expenditures by acting at sites primarily within the central nervous system (2–4). Obesity in humans and rodents is almost always associated with a resistance to, rather than a deficiency of, leptin (5–7). Resistance is associated with impaired transporter, receptor, postreceptor, and downstream neuronal circuitry functions in animal models of obesity (9–13). Leptin is transported across the blood-brain barrier (BBB) by a saturable transporter (8), and impaired transport can be acquired, may precede

receptor/postreceptor defects, worsens with increasing obesity, and is to some extent reversible (14–16). The relation between cerebrospinal fluid and serum levels of leptin in obese humans (17,18) suggests that defective BBB transport accounts for more of the overall resistance to leptin than the receptor/postreceptor defects (19).

The obesity-related defect in leptin BBB transport has two aspects (10). First, circulating substances cause an immediate impairment. Leptin itself, which is elevated in obesity, is likely one of these circulating substances. Second, an unidentified mechanism impairs transport in obese mice even when BBB transport is assessed by brain perfusion, a method that removes the immediate effects of blood-borne substances. Fasting or leptin administration can partially reverse these defects in leptin transport (16).

Starvation, like obesity, is accompanied by a decreased BBB transport rate of exogenous leptin (20). Whereas it is difficult to explain the evolutionary advantage of decreased leptin transport in obesity, an advantage is obvious in starvation. Decreasing the amount of the anorectic protein reaching the central nervous system should enhance the drive for seeking food. The mechanism of the starvation-induced impairment in transport is unknown but cannot be caused by leptin itself because its levels decrease with fasting (21).

Here, we postulate that triglycerides may underlie the impairment in BBB transport in both obesity and starvation. Triglycerides are decreased with fasting but are elevated with starvation and tend to be elevated with obesity. Supporting this hypothesis is the observation that mice with impaired triglyceride synthesis are protected against development of both diet-induced obesity and obesity-induced leptin resistance (22). Thus, hypertriglyceridemia could explain impaired transport of leptin across the BBB in both starvation and obesity.

## RESEARCH DESIGN AND METHODS

**Radioactive labeling of leptin.** Mouse recombinant leptin (a gift from Amgen, Thousand Oaks, CA) was radioactively labeled with <sup>125</sup>I (Amersham Pharmacia, Piscataway, NJ) by the lactoperoxidase method, and the I-Lep was purified on a column of G-10 Sephadex. Specific activity was ~100–125 Ci/g. **Measurement of leptin transport across the BBB in mice.** All studies were approved by the local animal care and use committee, were performed in an Association for Assessment and Accreditation of Laboratory Animal Care–approved facility, and used adult male CD-1 mice from our colony. Mice were anesthetized with urethane (4.0 g/kg i.p.), and the left jugular vein and right carotid artery were exposed. A total of 0.2 ml of Ringer's lactated solution (LR) with 1% BSA containing 10<sup>6</sup> cpm of I-Lep was injected into the jugular vein. Blood was collected from the carotid artery, and the whole brain was removed 10 min after the jugular injection, a time when the radioactivity represents intact I-Lep (8). Blood was centrifuged at 5,000g for 10 min at 4°C, and the serum was collected. The whole brain was cleaned of large vessels

From the <sup>1</sup>Department of Internal Medicine, Division of Geriatrics, Geriatric Research, Education, and Clinical Center, Veterans Affairs Medical Center, St. Louis University School of Medicine, St. Louis, Missouri; and the <sup>2</sup>Department of Pharmacology, Nagasaki University School of Medicine, Nagasaki, Japan.

Address correspondence and reprint requests to William A. Banks, 915 N. Grand Blvd., St. Louis, MO 63106. E-mail: bankswa@slu.edu.

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BBB, blood-brain barrier; DMOG, 1,2-dimethyl-3-oleoyl-rac-glycerol; DPOG, 1,2-dipalmitoyl-3-oleoyl-rac-glycerol; DSOG, 1,2-distearoyl-3-oleoyl-rac-glycerol; FFA, free fatty acid; LR, Ringer's lactated solution; MBEC, mouse brain endothelial cell.

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and weighed after discarding the pituitary and pineal gland. Levels of radioactivity in brain and serum were measured in a  $\gamma$ -counter, and brain/serum ratios ( $\mu\text{l/g}$ ) were calculated.

**Mouse brain perfusion studies.** Mice were anesthetized with urethane (4.0 g/kg i.p.). The thorax was opened, the heart was exposed, both jugulars were severed, and the descending thoracic aorta was clamped. A 26-gauge butterfly needle was inserted into the left ventricle of the heart, and the buffer of Zlokovic et al. (23) containing I-Lep [ $2(10)^6$  cpm/ml] was infused at a rate of 2 ml/min for 5 min (24). The exact counts per minute infused was determined on a 10- $\mu\text{l}$  aliquot of perfusion fluid. After perfusion, the vascular space of the brain was washed out by injecting 20 ml LR in <1 min through the left ventricle of the heart. The brain was removed as above, and brain/perfusion ratios were calculated. In other mice, 10% whole milk or 10% intralipid was included in the perfusate, and brains were collected after 1–5 min of perfusion without vascular washout. The unidirectional influx rate ( $K_i$ ) was calculated by regressing the brain/perfusion ratio against perfusion time.

**Mouse brain endothelial cell monolayers.** The protocol for isolating mouse brain endothelial cells (MBECs) was modified from that for rat brain endothelial cells (25–27). Brains from anesthetized CD-1 mice were cleaned of meninges and homogenized with a handheld scalpel. The homogenate was digested in a collagenase solution (1 mg/ml collagenase type 2 in 288 units/ml of DNase I; Sigma, St. Louis, MO) at 37°C for 1 h. Neurons, astrocytes, and Schwann cells were removed by centrifuging in Dulbecco's modified Eagle's medium solution (Sigma) containing 20% BSA. The partially purified mixture was digested again (1 mg/ml collagenase/dispase with 288 units/ml DNase I at 37°C for 30 min). Finally, the endothelial cells were purified on a 33% Percoll gradient (Amersham Biosciences) centrifuged at 1,000g for 10 min.

The MBECs were placed in culture dishes (Falcon) coated with 0.1 mg/ml collagen type 1 (Sigma) and 0.1 mg/ml fibronectin (Sigma) and incubated at 37°C with 5% CO<sub>2</sub> (27,28) in endothelial cell culture medium (20% plasma-derived serum [Quad Five, Ryegate, MT] containing 1 ng/ml basic fibroblast growth factor [Sigma] and Dulbecco's modified Eagle's medium) (25,29). Cell culture medium was changed every 2–3 days. MBECs were typically 70–80% confluent by day 7.

MBECs ( $4.0 \times 10^4$  cells/insert) cultured to 70–80% confluence were added to Transwell culture inserts (Coster, 24-well format, 3470) and cultured for 3 more days. Transwells had a culture plate (abluminal side) volume of 0.6 ml, an insert volume of 0.1 ml, and polyester membrane pores of 0.4  $\mu\text{m}$ . Transendothelial electrical resistance was used to confirm confluence of monolayers on the day of study. I-Lep was added to the luminal chamber with or without 10% milk. The abluminal chamber was sampled 1, 2.5, and 5 min after adding the I-Lep. The percentage of material transported per minute (PMT/min) was calculated with the following (30):

$$\text{PMT/min} = 100(\text{cpm in luminal sample})/[(\text{cpm in abluminal chamber})/t]$$

where  $t$  is time in minutes. The “cpm in abluminal chamber” was corrected by subtracting the cpm that had entered the luminal chamber and adding the cpm present in the MBEC inserts at the end of the experiment.

**Measurement of serum leptin levels.** The murine leptin radioimmunoassay used (Linco, St. Charles, MO) has a 50% cross-reactivity with rat leptin and no cross-reactivity with human or bovine leptin.

**Administration of milk and intralipid.** A total of 2.5 ml LR, whole milk, or intralipid (Pharmacia & UpJohn, Peapack, NJ) was injected intraperitoneally into CD-1 mice. I-Lep ( $10^6$  cpm in 0.2 ml LR) was injected 0.5–24 h later into the right jugular vein. Brain/serum ratios were calculated 10 min after intravenous injection of I-Lep as described above. In other mice, the uptake by brain of intravenous I-Lep was measured 4 h after the intraperitoneal injection of fat-free milk, whole milk, intralipid, or LR. In other mice, 0.2 ml fat-free milk, whole milk, intralipid, or LR containing  $10^6$  cpm of I-Lep was given intravenously 10 min before harvesting brain and blood.

**Preparation of triglyceride and free fatty acid emulsions.** Triglycerides or free fatty acid (FFA) and L- $\alpha$ -phosphatidylcholine (all from Sigma) were each dissolved in chloroform, mixed, and dried under a stream of nitrogen gas. Zlokovic's buffer was added, and the material was vigorously mixed, homogenized, and alternatively frozen in liquid nitrogen and thawed in a warm water bath for 12 cycles. Material was either immediately diluted to the desired concentration with LR containing I-Lep and injected intravenously or stored at  $-20^\circ\text{C}$  for use within 48 h. Oleate was purchased in liquid form, dried, and used immediately after dissolving in chloroform. On the day of study, anesthetized mice received intravenous injections of 0.2 ml LR containing 1% BSA and  $10^6$  cpm I-Lep with or without an FFA or triglyceride emulsion. Carotid artery blood and brains were obtained 10 min later, and results were expressed as brain/serum ratios.

**Administration of gemfibrozil.** Mice were weighed and fed 1 ml/kg vegetable oil with or without gemfibrozil (1 g/kg) twice per day for 5 days. On the morning after the day of the last dose, mice were anesthetized and given

intravenous I-Lep, and brain and arterial blood was collected 10 min later as described above. Triglyceride levels were measured on arterial serum with a kit (Sigma).

**Diet-induced obesity.** Mice were weighed and kept on regular food (4.5% fat, 5001 Rodent Diet; PMI Nutrition International, Brentwood, MO) or switched to breeder food (10% fat, Teklad Mouse Breeder Diet; Harlan Teklad, Madison, WI) for an average of 17 weeks. Brain and blood samples were collected 10 min after intravenous I-Lep as described above. Triglyceride levels were measured on serum. This experiment was repeated, except that each dietary group was randomized into 16-h fasted and nonfasted groups.

**Statistical analysis.** Means are reported with their standard errors and  $n$ . Two groups were compared by a  $t$  test. More than two groups were compared by ANOVA followed by a Newman-Keuls post hoc test. Regression lines were computed by the least-squares method, and their slopes were compared with the software package in Prism 4.0 (GraphPad, San Diego, CA).

## RESULTS

Starvation for 48 h impaired transport of intravenously administered I-Lep, decreasing brain/serum ratios from  $19.1 \pm 1.28 \mu\text{l/g}$  ( $n = 10$ ) to  $15.1 \pm 1.01 \mu\text{l/g}$  ( $n = 8$ ) (Fig. 1A;  $P < 0.05$ ). About 10  $\mu\text{l/g}$  of the brain/serum ratio represents vascular space, so the decrease in I-Lep uptake was from  $\sim 9.1$  to 5.1, or  $\sim 44\%$ . In contrast to intravenous injection results, brain perfusion found no difference in leptin transport between fed and 48-h starved mice (Fig. 1B). Starvation for 48 h increased serum triglyceride levels ( $165 \pm 9 \text{ mg/dl}$ ,  $n = 5$ ) in comparison to nonfasted ( $135 \pm 10$ ,  $n = 6$ ,  $P < 0.05$ ) or 16-h fasted ( $96 \pm 7$ ,  $n = 7$ ,  $P < 0.001$ ) mice (Fig. 1C).

The effect of triglycerides on I-Lep transport was tested by injecting bovine whole milk (98% of fat content being triglycerides) or intralipid, a source of plant triglycerides and FFA, into the peritoneal cavity. Brain/serum ratios are expressed relative to the time-matched mice injected with LR (Fig. 2A,  $n = 5$ –6 mice/point). Milk produced an immediate long-lasting impairment in leptin transport, whereas intralipid had no statistically significant effect. Two-way ANOVA showed an effect for treatment [ $F(2,69) = 30.5$ ,  $P < 0.001$ ], time [ $F(6,69) = 5.62$ ,  $P < 0.001$ ], and interaction [ $F(12,69) = 2.86$ ,  $P < 0.005$ ]. Newman-Keuls post hoc test showed that the 30-min, 2-h, and 4-h values for milk differed from the time-matched controls (all at  $P < 0.01$ ). Two-way ANOVA showed that serum triglyceride levels (Fig. 2B) were elevated after intraperitoneal injection of milk with significant effects for treatment [ $F(1,55) = 21.4$ ,  $P < 0.0001$ ] and time [ $F(1,55) = 6.26$ ,  $P < 0.0005$ ] but not interaction. For serum leptin levels, two-way ANOVA showed a significant effect for treatment [ $F(1,56) = 10.1$ ,  $P < 0.005$ ], a trend for time [ $F(1,56) = 2.25$ ,  $P = 0.07$ ], and no effect for interaction (Fig. 2C). Newman-Keuls post hoc test found no time-matched differences between mice treated with milk versus LR for either triglycerides or leptin.

Brain perfusion found that whole milk, but not intralipid, inhibited the transport of I-Lep across the BBB (Fig. 3). Transport rates for I-Lep perfused in buffer ( $K_i = 2.74 \pm 0.79 \mu\text{l} \cdot \text{g}^{-1} \cdot \text{min}^{-1}$ ,  $n = 18$ ,  $r = 0.655$ ,  $P < 0.005$ ) or intralipid ( $K_i = 2.29 \pm 0.85 \mu\text{l} \cdot \text{g}^{-1} \cdot \text{min}^{-1}$ ,  $n = 13$ ,  $r = 0.631$ ,  $P < 0.05$ ) were not different.  $K_i$  for I-Lep in milk was not measurable ( $n = 18$ ,  $r = 0.217$ ,  $P > 0.4$ ) and differed from buffer [ $F(1,31) = 6.64$ ,  $P < 0.05$ ] or intralipid [ $F(1,26) = 4.51$ ,  $P < 0.05$ ] perfusions.

Whole milk inhibited leptin transport across MBEC monolayers (Fig. 4), decreasing the percentage of material

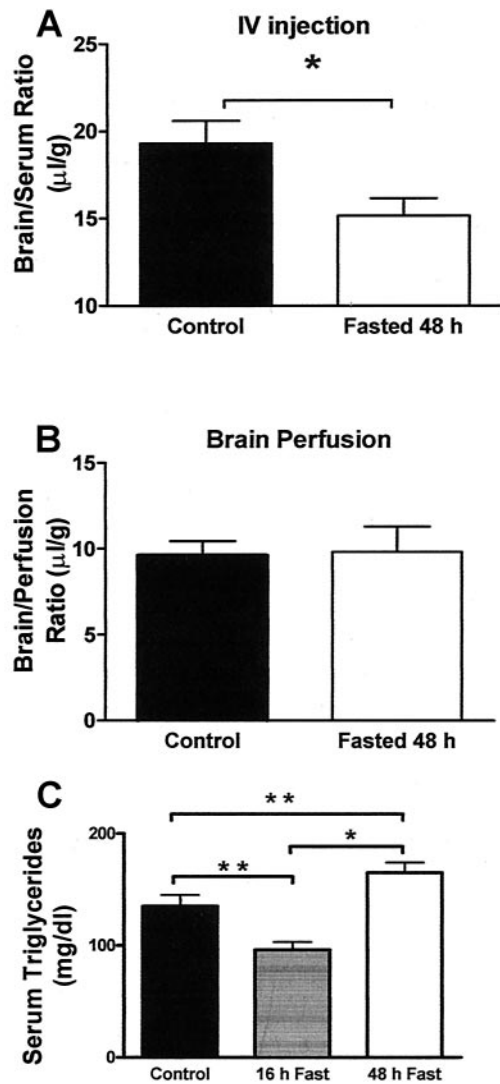


FIG. 1. Starvation-induced inhibition of leptin transport across the BBB is caused by a circulating factor. *A*: Replication of classic work (20) showing that the leptin transporter is inhibited with starvation. *B*: With brain perfusion, a method that negates the effect of circulating substances, the transporter returns to normal. *C*: Triglyceride levels in serum in control mice, mice fasted for 16 h, and mice fasted for 48 h. IV, intravenous. \* $P < 0.05$ ; \*\* $P < 0.01$ .

transported per minute from  $(3.42 \pm 0.085)10^{-4}$  to  $(3.05 \pm 0.06)10^{-4}$  ( $n = 24/\text{group}$ ,  $P < 0.001$ ).

Nonfat milk was compared with whole milk and intralipid by injecting animals intraperitoneally 4 h before the intravenous injection of I-Lep or by coinjecting them with the intravenous I-Lep. Only whole milk had an effect on I-Lep uptake (Fig. 5).

Triglycerides (7.2 mg/mouse, an amount equal to the total triglyceride content in 0.2 ml milk) were included in the intravenous injection of I-Lep given 10 min before collection of brain and blood. Three triglycerides (triolein, 1,2-dipalmitoyl-3-oleoyl-rac-glycerol [DPOG], and 1,2-distearoyl-3-oleoyl-rac-glycerol [DSOG]) each inhibited I-Lep transport (all  $P < 0.001$ ), but 1,2-dimyristoyl-3-oleoyl-rac-glycerol (DMOG) had no effect (Fig. 6A). An inverse linear relation existed between the log dose of intravenous triolein and brain uptake of I-Lep [Fig. 6B,  $n = 7-8$  mice/dose,  $Y = 72.3 - 12.0X$ ,  $r = (-0.827)$ ,  $n = 7$ ,  $P < 0.05$ ].

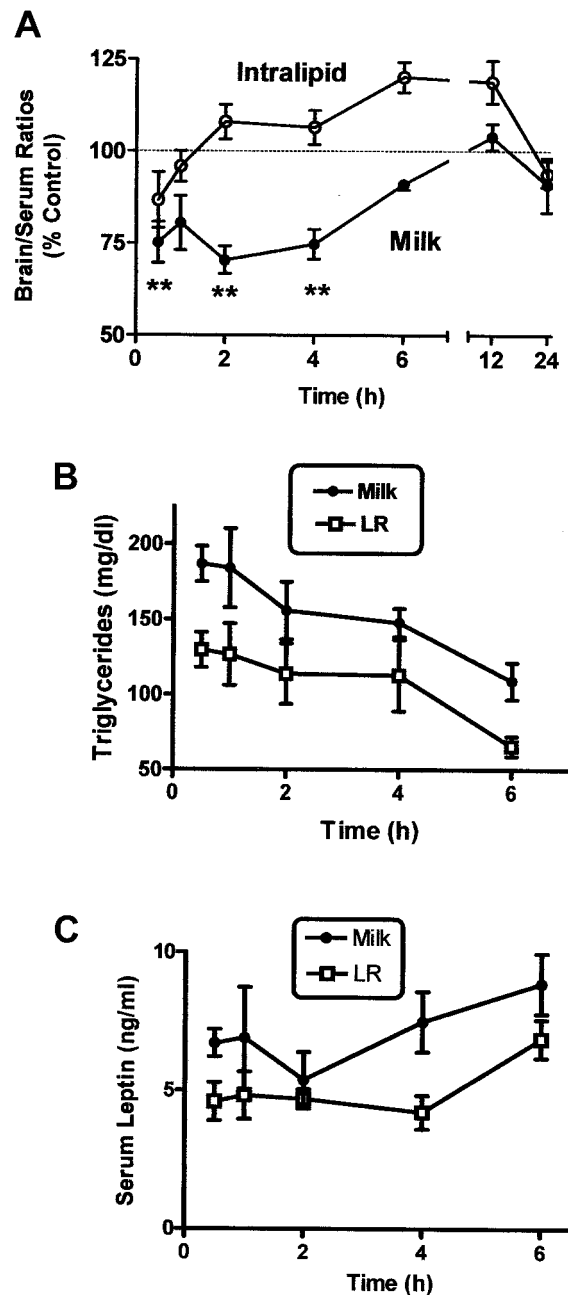


FIG. 2. Leptin resistance to BBB transport is induced by milk fats. *A*: Intraperitoneal injection of whole milk, but not intralipid, induced an impairment in leptin transport across the BBB. Intraperitoneal injection of milk increased serum triglycerides (*B*) and serum leptin levels (*C*) by ~40%. \*\* $P < 0.01$ .

The FFA derivable form triolein, DPOG, and DSOG were tested for their ability to inhibit I-Lep transport (Fig. 6A). Doses tested of palmitate (0.4 mg/mouse), stearate (0.4 mg/mouse), and oleate (0.72 mg/mouse) were those estimated to produce a level in blood 10–20 times higher than the level seen for FFAs in starvation. FFAs were injected intravenously with I-Lep, and brain and serum samples were collected 10 min later. These FFAs had no effect on leptin transport. Oleate tested at the dose of 7.2 mg/mouse, the dose at which triolein was effective, was without effect.

Obesity was induced by placing male CD-1 mice on breeder food (10% fat, Teklad Mouse Breeder Diet; Harlan

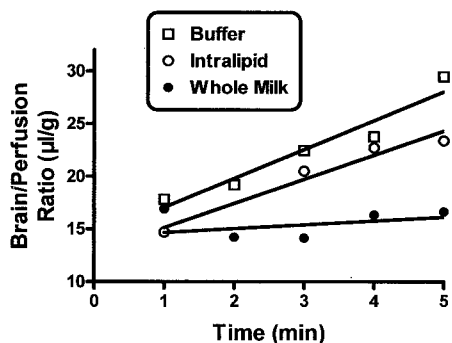


FIG. 3. Brain perfusion with intralipid and milk. The slope of the relation between brain/perfusion ratios and time measures  $K_1$  for I-Lep. Intralipid had no effect on BBB transport, but whole milk prevented any measurable transport.

Teklad) for 17 weeks and comparing them to littermates left on a regular food (4.5% fat, 5001 Rodent Diet; PMI Nutrition International). Mice fed breeder food weighed ~44% more than mice fed regular food. In the initial study (experiment 1), 16 mice were used per group. This study was repeated (experiment 2) with half the mice fasted for 16 h ( $n = 8/\text{group}$ ). With the high-fat diet, serum triglycerides increased and leptin transport decreased. Fasting decreased serum triglycerides and increased leptin transport in both mice fed breeder food and mice fed regular food (Fig. 7). Triglyceride levels and leptin transport were inversely related among these groups:  $Y = 22.6 - 0.044X$ ,  $r = -0.860$ ,  $P < 0.05$ .

Short-term administration of gemfibrozil reduced triglyceride levels to  $<100$  mg/dl in four of six mice fed regular food (Fig. 8A,  $n = 7$  control,  $n = 6$  vehicle  $P < 0.05$ ). These four mice had a statistically significant increase in leptin transport in comparison to mice fed vehicle only (Fig. 8B,  $P < 0.05$ ). A statistically significant correlation existed between the means for serum triglycerides and brain/serum ratios for leptin (Fig. 8C;  $r = 1.0$ ,  $n = 3$ ,  $P < 0.01$ ). A decrease in body weight seen in these four mice was not statistically significant (Fig. 8D).

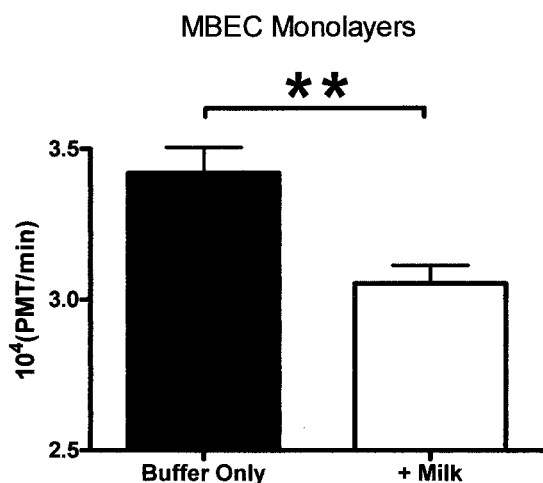


FIG. 4. In vitro assessment of milk in a monolayer model of the BBB using cultured mouse brain endothelial cells. Addition of milk to the buffer inhibited transport of leptin. PMT, percentage of material transported.  $**P < 0.001$ .

## DISCUSSION

Obesity is associated with leptin resistance caused by impaired leptin transport across the BBB, defects in leptin-receptor signaling, and blockades in downstream neuronal circuitries. The inability of obese mice to respond to peripherally administered leptin while responding to centrally administered leptin is likely caused by a defect in leptin transport across the BBB. It is unclear what causes defective transport of leptin in either obesity or starvation. Because serum triglycerides are elevated in both starvation and obesity, we postulated that triglycerides inhibit leptin transport across the BBB. Here, we showed that starvation-induced inhibition of leptin transport was caused by a circulating factor; that the fat component of milk (which is 98% triglycerides) as well as specific triglycerides could induce inhibition of leptin transport across the BBB in vivo, in situ, and in vitro; that the FFAs comprising those triglycerides were ineffectual; that manipulation of triglyceride levels with diet or fasting in normal or obese mice had an inverse effect on leptin transport; and that reduction of triglycerides by pharmacological intervention reversed the impairment in leptin transport. Taken together, these findings show that triglycerides directly inhibit the transport of leptin across the BBB and so could be a major cause of leptin resistance at the BBB.

We fasted mice for 48 h to determine whether starvation impairs the transport of intravenously administered I-Lep. We confirmed that short-term fasting decreased serum triglyceride levels, whereas 48 h of fasting (starvation) increased them. The level of reduction in the brain uptake of intravenous administered I-Lep was almost identical to the results found by others (20). That group further showed that longer fasts progressively impaired leptin transport across the BBB to the point of total inhibition after 5 days of starvation. This inhibition of leptin transport and the accompanying decrease in levels of leptin in the blood (21) are likely adaptive because they would reduce the anorectic signal in starvation.

In contrast to intravenous injection, brain perfusion

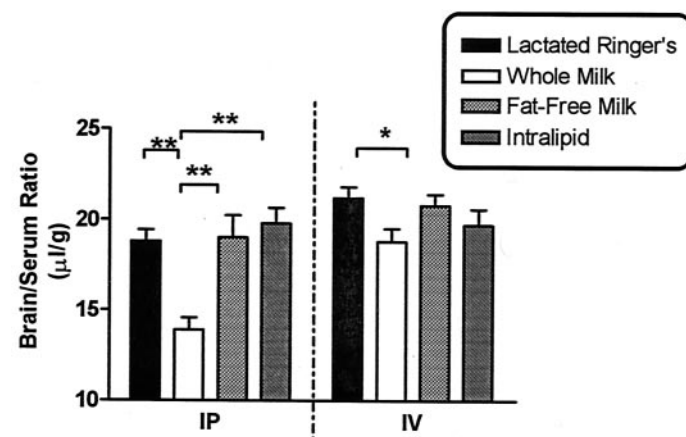
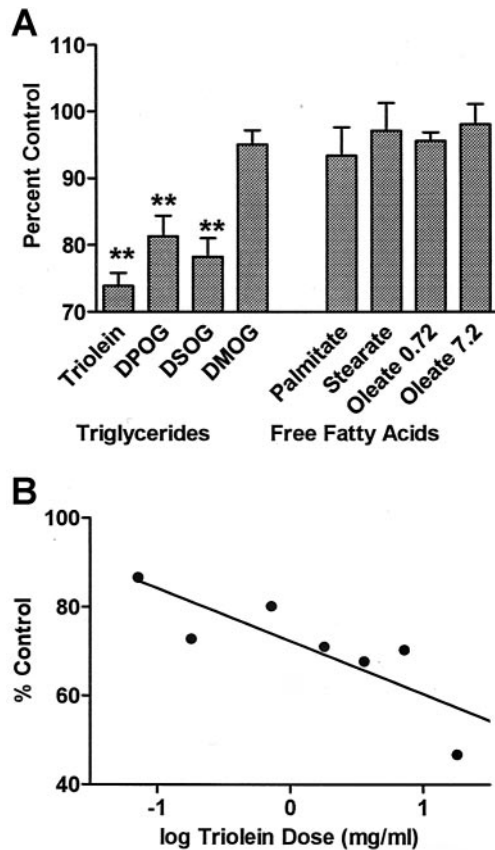


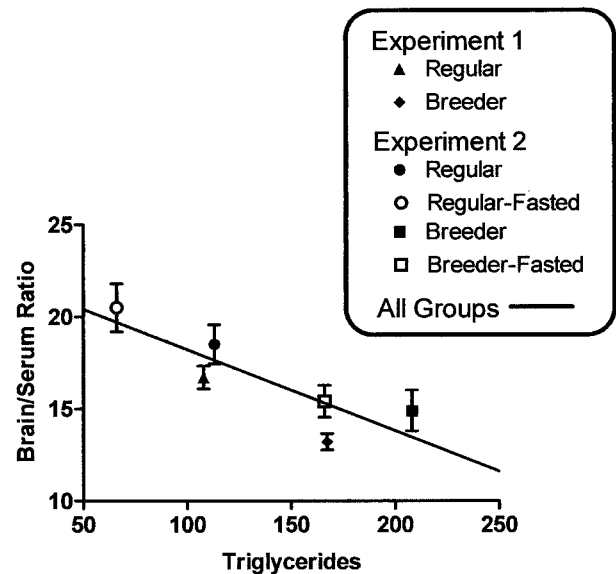
FIG. 5. Intraperitoneal (IP) and intravenous (IV) milk fat inhibits leptin transport. With IP injection, fat-free milk and intralipids were ineffective, whereas whole milk inhibited leptin transport. Milk given intravenously (0.2 ml milk administered intravenously with the I-Lep, with brain and serum samples collected 10 min later) was immediately effective in reducing I-Lep transport, whereas fat-free milk and intralipid were not.  $*P < 0.05$ ;  $**P < 0.01$ .



**FIG. 6.** Triglycerides and leptin transport. **A:** Triglycerides but not FFAs induce resistance to leptin transport across the BBB. Triglycerides or FFAs were given intravenously with the I-Lep and brain and blood samples collected 10 min later. Three triglycerides (triolein, DPOG, and DSOG) inhibited leptin transport across the BBB. DMOG was ineffective in mice. Triglycerides were given at a dose of 7.2 mg/mouse to replicate the dose of total triglycerides in a volume of 0.2 ml whole milk. Palmitate (0.4 mg/mouse), stearate (0.4 mg/mouse), and oleate (0.72 mg/mouse) were ineffective at doses calculated to produce blood levels exceeding those seen in starvation. Oleate was also ineffective at the doses at which triolein was effective (7.2 mg/mouse).  $**P < 0.01$ . **B:** Triolein has a dose-dependent effect on I-Lep transport. Triolein and I-Lep were injected together, and brain and blood samples were collected 10 min later.

found no difference in leptin uptake between starved and fed mice. Because the intravenous and brain perfusion methods usually give similar results except when a circulating factor has an acutely reversible effect on transport (31–33), the results show that starvation inhibits leptin transport by releasing a blood-borne factor.

We more directly tested triglycerides by injecting bovine whole milk or intralipid into the peritoneal cavity. The fat in milk is 98% triglycerides (34), whereas the fat in intralipid is a soybean oil-based source of triglycerides containing the essential FFAs linolenic and linoleic acid, purified egg phospholipids, and glycerol. Milk increased serum triglyceride and leptin levels by ~40% and produced an immediate long-lasting impairment in leptin transport across the BBB. Serum triglycerides showed a time-dependent decline during the course of the study in both milk- and vehicle-injected animals, probably related to diurnal rhythm. However, at those times when leptin transport was inhibited, serum triglycerides in milk-injected animals were higher than the vehicle-injected animals' highest value (baseline). The increase in serum leptin levels was likely produced by the mouse because pasteurization



**FIG. 7.** Effect of high-fat (breeder food) diet and 16 h of fasting on triglyceride levels and leptin transport. I-Lep was injected intravenously, and brain and blood samples were collected 10 min later. Triglyceride levels were measured in serum. The relation between triglyceride levels and brain/serum ratios was statistically significant ( $r = -0.860$ ,  $n = 6$ ,  $P < 0.05$ ). The regular and breeder groups were tested twice: first as the initial experiment (experiment 1) and again as controls to the fasting groups (experiment 2).

significantly reduces leptin levels in milk (35), and our immunoassay was species specific. The increase in serum leptin from ~4.5 to 6.5 ng/ml during the period of leptin inhibition is likely too low to explain the inhibition in leptin transport. Previous work shows that this would result in only about a 10% decrease in the leptin transport rate (33). Additionally, leptin levels were highest at 6 h, a time when transport was no longer significantly inhibited. In fact, the 6-h serum leptin level for vehicle-treated mice was a little higher than the serum leptin level in milk-treated mice at 2 h, the time of greatest inhibition in leptin transport. Milk also inhibited leptin transport in the in situ brain perfusion model and in the in vitro brain monolayer model of the BBB, conditions where leptin secretion could not occur.

In comparison to milk, intralipid was without effect, suggesting that plant triglycerides and essential FFAs do not inhibit leptin transport. Milk given intravenously was immediately effective at less than one-tenth the intraperitoneal dose. The immediacy of effect after intravenous injection suggests that triglycerides rather than a degradation product (e.g., FFAs) affected transport. Nonfat milk, which contains the same concentration of proteins and phospholipids as whole milk and has only the triglycerides removed (34), was without effect. These results show that inhibition was not caused by leptin remaining in pasteurized milk (35,36). They also show that animal-derived triglycerides impair leptin transport across the BBB, but not essential FFAs, plant-derived triglycerides, or milk proteins.

We directly tested the ability of triglycerides to inhibit leptin transport across the BBB. Three of four commercially available triglycerides (triolein, DPOG, and DSOG) inhibited uptake of I-Lep when injected intravenously at a dose that equaled the total triglyceride content of milk

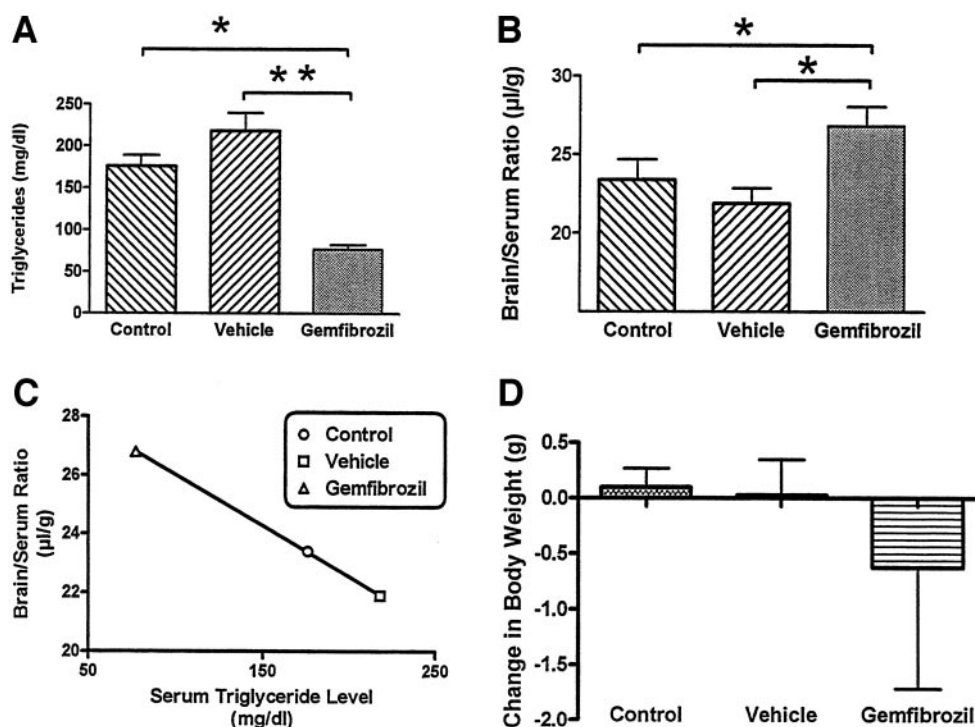


FIG. 8. Effect of gemfibrozil on serum triglyceride levels and leptin transport. I-Lep was injected intravenously, brain and blood samples were collected 10 min later, and triglyceride levels were measured in serum. **A:** In comparison to mice that received the vegetable oil vehicle only, gemfibrozil responders (four of six treated mice) had lower triglyceride levels ( $**P < 0.01$ ). **B:** Gemfibrozil responders had higher leptin transport rates than vehicle ( $*P < 0.05$ ) or control ( $*P < 0.05$ ) mice. **C:** A significant correlation ( $P < 0.01$ ) existed between brain/serum ratios for leptin and serum triglyceride levels for the three groups. **D:** The weight loss for the gemfibrozil group was not statistically significant.

(Fig. 6A). A dose-response curve suggests that, at least in the case of triolein, lower doses are also effective. DMOG, the triglyceride that did not inhibit leptin transport, illustrates that the *sn-1* position is important for the inhibitory effect. Myristate, as a medium-chain FFA, is only produced in by mammary alveolar cells; therefore, triglycerides containing it may not reflect diet or obesity (37). Additionally, it would not be expected to circulate in significant amounts in blood. These results suggest that leptin transport will be inhibited by triglycerides endogenous to blood.

We ruled out the possibility that FFAs hydrolyzed from the triglycerides were inhibiting leptin transport. The FFAs (palmitate, stearate, and oleate) that could be hydrolyzed from the triglycerides were without effect at doses that would have produced blood levels higher than those seen in starvation. We also tested oleate at the same dose as triolein, but found it was without effect. Because the molecular weight of triolein is only  $\sim 75\%$  fatty acid with the remainder comprised of the glycerol backbone, we tested oleate at a molarity at least 30% higher than could be achieved with total hydrolyzation of triolein. This shows that the triglycerides themselves and not the FFAs derived from them are responsible for inhibiting leptin transport.

We tested the pathophysiological relevance of hypertriglyceridemia by studying the effects of dietary-induced obesity on the relation between triglycerides and I-Lep uptake. We also tested the ability of a 16-h fast to affect the relation between triglycerides and I-Lep uptake in these groups of mice. As Fig. 7 illustrates, diet-induced obesity

increased triglycerides and reduced I-Lep uptake by the brain. In both lean and diet-induced obese mice, fasting reduced triglycerides and increased I-Lep uptake by the brain.

Gemfibrozil is selective for reduction of serum triglyceride levels and is used clinically for the treatment of hypertriglyceridemia. Short-term administration of gemfibrozil reduced triglyceride levels to  $< 100$  mg/dl in four of six mice (Fig. 8). These responders had a statistically significant increase in leptin transport in comparison to mice fed vehicle ( $P < 0.05$ ). This showed that reduction of triglyceride levels by pharmacological treatment could enhance leptin transport across the BBB.

These results show that serum triglycerides have a rapid and immediate effect on the transport of leptin. As such, they explain the inhibition in leptin transport seen with starvation. They also likely contribute to the inhibition seen with obesity. Triglycerides could produce their effect on leptin transport by binding leptin in the circulation or by acting directly on the leptin transporter. Other BBB transporters are known to be regulated by uncompetitive and noncompetitive mechanisms (38,39), and leptin transport is altered by  $\alpha_1$ -adrenergic agonists, glucose, and insulin (40,41). It may be that the leptin transporter possesses a regulatory site controlled by triglycerides.

The ability of triglycerides to inhibit leptin transport into the brain completes a negative feedback loop between leptin action and triglycerides. Leptin promotes triglyceride hydrolysis and FFA oxidation and inhibits FFA synthesis (42,43,44), therefore decreasing triglyceride levels.

The importance of leptin in reducing triglyceride levels

is dramatically illustrated in patients with lipodystrophy and lipatrophy. These patients have little or no fat mass and, as a result, have little or no leptin. They also have very severe hypertriglyceridemia that is reversed by treatment with leptin (45). The ability of triglycerides to induce leptin resistance would counter the leptin-induced shift toward use of triglycerides as an energy source and so help to conserve fat stores. This would make evolutionary sense because hypertriglyceridemia has probably more often represented starvation than obesity. Healthy baboons living in the wild have fat stores and serum leptin levels that are a fraction of those seen in Western humans and laboratory animals (46), but when supplied with abundant calories, develop a condition resembling the metabolic syndrome X (47), including the development of hyperlipidemia (48). These studies in wild baboons are consistent with the hypothesis that ancestral levels of leptin were much lower than those seen in Western civilization and that starvation was a more probable threat than obesity.

The usefulness of leptin resistance in obesity is less clear than its obvious utility in starvation. Starvation-induced hypertriglyceridemia may have been so dominant an evolutionary pressure that leptin resistance induced by obesity-related hypertriglyceridemia was never selected against. Alternatively, it may be that the anorectic effect of leptin must be overridden to maintain an adequate intake of water-soluble vitamins, minerals, electrolytes, and other substances less efficiently stored than fat.

Our results also provide a mechanism to explain previous findings of why mice unable to synthesize triglycerides are more sensitive to leptin (22). Mice lacking acyl coenzyme A:diacylglycerol acyltransferase 1, a critical enzyme needed to synthesize triglycerides, are more sensitive to infusions of leptin. Without this enzyme, obesity did not develop in a strain of mice normally resistant to leptin but did in a strain that is leptin deficient. This shows that leptin is critical to the mechanism by which lack of triglycerides protects from diet-induced obesity. The results presented here provide one mechanism by which lowering triglycerides can increase leptin sensitivity.

In conclusion, these studies show that serum triglycerides impair the ability of the BBB to transport leptin. Triglycerides are likely a major cause of the leptin resistance seen in both starvation and obesity (9,10,16,20). Lowering triglycerides may be therapeutically useful in enhancing the effects of leptin on weight loss.

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#### REFERENCES

- Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM: Positional cloning of the mouse *obese* gene and its human homologue. *Nature* 372:425–432, 1994
- Halaas JL, Gajiwala KS, Maffei M, Cohen SL, Chait BT, Rabinowitz D, Lallone RL, Burley SK, Friedman JM: Weight-reducing effects of the plasma protein encoded by the *obese* gene. *Science* 269:543–546, 1995
- Campfield LA, Smith FJ, Guisez Y, Devos R, Burn P: Recombinant mouse OB protein: evidence for a peripheral signal linking adiposity and central neural networks. *Science* 269:546–549, 1995
- Pelleymounter MA, Cullen MJ, Baker MB, Hecht R, Winters D, Boone T, Collins F: Effects of the *obese* gene product on body weight regulation in *ob/ob* mice. *Science* 269:540–543, 1995
- Considine RV, Sinha MK, Heiman ML, Kriauciunas A, Stephens TW, Nyce MR, Ohannesian JP, Marco CC, McKee LJ, Bauer TL, Caro JF: Serum immunoreactive-leptin concentrations in normal-weight and obese humans. *N Engl J Med* 334:292–295, 1996
- Pelleymounter MA, Cullen MJ, Healy D, Hecht R, Winters D, McCaleb M: Efficacy of exogenous recombinant murine leptin in lean and obese 10- to 12-mo-old female CD-1 mice. *Am J Physiol* 275:R950–R959, 1998
- Heymsfield SB, Greenberg AS, Fujioka K, Dixon RM, Kushner R, Hunt T, Lubina JA, Patane J, Self B, Hunt P, McCamish M: Recombinant leptin for weight loss in obese and lean adults: a randomized, controlled, dose-escalation trial. *JAMA* 282:1568–1575, 1999
- Banks WA, Kastin AJ, Huang W, Jaspan JB, Maness LM: Leptin enters the brain by a saturable system independent of insulin. *Peptides* 17:305–311, 1996
- Hileman SM, Pierroz DD, Masuzaki H, Bjorbaek C, El Haschimi K, Banks WA, Flier JS: Characterization of short isoforms of the leptin receptor in rat cerebral microvessels and of brain uptake of leptin in mouse models of obesity. *Endocrinology* 143:775–783, 2002
- Banks WA, DiPalma CR, Farrell CL: Impaired transport of leptin across the blood-brain barrier in obesity. *Peptides* 20:1341–1345, 1999
- El Haschimi K, Pierroz DD, Hileman SM, Bjorbaek C, Flier JS: Two defects contribute to hypothalamic leptin resistance in mice with diet-induced obesity. *J Clin Invest* 105:1827–1832, 2000
- Yamashita T, Murakami T, Iida M, Kuwajima M, Shima K: Leptin receptor of Zucker fatty rat performs reduced signal transduction. *Diabetes* 46:1077–1080, 1997
- Wilsey J, Zolotukhin S, Prima V, Scarpace PJ: Central leptin gene therapy fails to overcome leptin resistance associated with diet-induced obesity. *Am J Physiol* 285:R1011–R1020, 2003
- van Heek M, Compton DS, France CF, Tedesco RP, Fawzi AB, Graziano MP, Sybertz EJ, Strader CD, Davis J: Diet-induced obese mice develop peripheral, but not central, resistance to leptin. *J Clin Invest* 99:385–390, 1997
- Halaas JL, Boozer C, Blair-West J, Fidathusein N, Denton DA, Friedman JM: Physiological response to long-term peripheral and central leptin infusion in lean and obese mice. *Proc Natl Acad Sci U S A* 94:8878–8883, 1997
- Banks WA, Farrell CL: Impaired transport of leptin across the blood-brain barrier in obesity is acquired and reversible. *Am J Physiology* 285:E10–E15, 2003
- Caro JF, Kolaczynski JW, Nyce MR, Ohannesian JP, Opentanova I, Goldman WH, Lynn RB, Zhang PL, Sinha MD, Considine RV: Decreased cerebrospinal-fluid/serum leptin ratio in obesity: a possible mechanism for leptin resistance. *Lancet* 348:159–161, 1996
- Schwartz MW, Peskind E, Raskind M, Boyko EJ, Porte D Jr: Cerebrospinal fluid leptin levels: relationship to plasma levels and adiposity in humans. *Nat Med* 2:589–593, 1996
- Banks WA: Is obesity a disease of the blood-brain barrier? Physiological, pathological, and evolutionary considerations. *Current Pharmaceutical Design* 9:801–809, 2003
- Kastin AJ, Akerstrom V: Fasting, but not adrenalectomy, reduces transport of leptin into the brain. *Peptides* 21:679–682, 2000
- Kolaczynski JW, Considine RV, Ohannesian J, Marco C, Opentanova I, Nyce MR, Myint M, Caro JF: Responses of leptin to short-term fasting and refeeding in humans: a link with ketogenesis but not ketones themselves. *Diabetes* 45:1511–1515, 1996
- Chen HC, Smith SJ, Ladha Z, Jensen DR, Ferreira LD, Pulawa LK, McGuire JG, Pitas RE, Eckel RH, Farese RV Jr: Increased insulin and leptin sensitivity in mice lacking acyl CoA:diacylglycerol acyltransferase 1. *J Clin Invest* 109:1049–1055, 2002
- Zlokovic BV, Lipovac MN, Begley DJ, Davson H, Rakic L: Slow penetration of thyrotropin-releasing hormone across the blood-brain barrier of an in situ perfused guinea pig brain. *J Neurochem* 51:252–257, 1988
- Shayo M, McLay RN, Kastin AJ, Banks WA: The putative blood-brain barrier transporter for the  $\beta$ -amyloid binding protein apolipoprotein J is saturated at physiological concentrations. *Life Sci* 60:L115–L118, 1996
- Abbott NJ, Hughes CCW, Revest PA, Greenwood J: Development and characterization of a rat brain capillary endothelial culture: towards an in vitro blood-brain barrier. *J Cell Sci* 103:23–37, 1992
- Deli MA, Joo F: Cultured vascular endothelial cells of the brain. *Keio J Med* 45:183–198, 1996
- Kis B, Kaiya H, Nishi R, Deli MA, Abraham CS, Yanagita T, Isse T, Gotoh

- S, Kobayashi H, Wada A, Niwa M, Kangawa K, Greenwood J, Yamashita H, Ueta Y: Cerebral endothelial cells are a major source of adrenomedullin. *J Neuroendocrinology* 14:283–293, 2002
28. Domotor E, Sipos I, Kittel A, Abbott NJ, Adam-Vizi V: Improved growth of cultured brain microvascular endothelial cells on glass coated with a biological matrix. *Neurochem Int* 33:473–478, 1998
  29. Demeuse P, Kerkhofs A, Struys-Ponsar C, Knoops B, Remacle C, van den Bosch de Aguilar P: Compartmentalized coculture of rat brain endothelial cells and astrocytes: a syngenic model to study the blood-brain barrier. *J Neurosci Methods* 121:21–31, 2002
  30. Maresh GA, Maness LM, Zadina JE, Kastin AJ: In vitro demonstration of a saturable transport system for leptin across the blood-brain barrier. *Life Sci* 69:67–73, 2001
  31. Smith QR, Momma S, Aoyagi M, Rapoport SI: Kinetics of neutral amino acid transport across the blood-brain barrier. *J Neurochem* 49:1651–1658, 1987
  32. Zlokovic BV, Begley DJ, Djuricic BM, Mitrovic DM: Measurement of solute transport across the blood-brain barrier in the perfused guinea pig brain: method and application to N-methyl- $\alpha$ -aminoisobutyric acid. *J Neurochem* 46:1444–1451, 1986
  33. Banks WA, Clever CM, Farrell CL: Partial saturation and regional variation in the blood to brain transport of leptin in normal weight mice. *Am J Physiol* 278:E1158–E1165, 2000
  34. Christie WW: Composition and structure of milk lipids. In *Advanced Dairy Chemistry. Volume. 2: Lipids*. Fox PF, Ed. Chapman and Hall, 1994, p. 1–36
  35. Resto M, O'Connor D, Leef K, Funanage V, Spear M, Locke R: Leptin levels in preterm human breast milk and infant formula. *Pediatrics* 108:E15, 2001
  36. Smith-Kirwin SM, O'Connor DM, De Johnston J, Lancey ED, Hassink SG, Funanage VL: Leptin expression in human mammary epithelial cells and breast milk. *J Clin Endocrinol Metab* 83:1810–1813, 1998
  37. Neville MC, Picciano MF: Regulation of milk lipid secretion and composition. *Ann Rev Nutr* 17:159–184, 1997
  38. Banks WA, Kastin AJ: Modulation of the carrier-mediated transport of the Tyr-MIF-1 across the blood-brain barrier by essential amino acids. *J Pharmacol Exp Ther* 239:668–672, 1986
  39. Banks WA, Kastin AJ, Fasold MB: Differential effect of aluminum on the blood-brain barrier transport of peptides, technetium and albumin. *J Pharmacol Exp Ther* 244:579–585, 1988
  40. Banks WA: Enhanced leptin transport across the blood-brain barrier by  $\alpha$ 1-adrenergic agents. *Brain Res* 899:209–217, 2001
  41. Kastin AJ, Akerstrom V: Glucose and insulin increase the transport of leptin through the blood-brain barrier in normal mice but not in streptozotocin-diabetic mice. *Neuroendocrinology* 73:237–242, 2001
  42. Steinberg GR, Bonen A, Dyck DJ: Fatty acid oxidation and triacylglycerol hydrolysis are enhanced after chronic leptin treatment in rats. *Am J Physiol* 282:E593–E600, 2002
  43. Reidy SP, Weber JM: Accelerated substrate cycling: a new energy-wasting role for leptin in vivo. *Am J Physiol* 282:E312–E317, 2002
  44. Matsuoka N, Ogawa Y, Masuzaki H, Ebihara K, Aizawa-Abe M, Satoh N, Ishikawa E, Fujisawa Y, Kosaki A, Yamada K, Kuzuya H, Nakao K: Decreased triglyceride-rich lipoproteins in transgenic skinny mice overexpressing leptin. *Am J Physiol Endocrinol Metab* 280:E334–E339, 2001
  45. Oral EA, Simha V, Ruiz E, Andewelt A, Premkumar A, Snell P, Wagner AJ, DePaoli AM, Reitman ML, Taylor SI, Gorden P, Garg A: Leptin-replacement therapy for lipodystrophy. *N Engl J Med* 346:570–578, 2002
  46. Banks WA, Phillips-Conroy JE, Jolly CJ, Morley JE: Serum leptin levels in wild and captive populations of baboons (*Papio*): implications for the ancestral role of leptin. *J Clin Endocrinol Metab* 86:4315–4320, 2001
  47. Hansen BC, Saye J, Wennogle LP: *The Metabolic Syndrome X: Convergence of Insulin Resistance, Glucose Intolerance, Hypertension, Obesity, and Dyslipidemia: Searching for the Underlying Defects*. New York, New York Academy of Sciences, 1999, p. 1–336
  48. Banks WA, Altmann J, Sapolsky RM, Phillips-Conroy JE, Morley JE: Serum leptin levels as a marker for a syndrome X-like condition in wild baboons. *J Clin Endocrinol Metab* 88:1234–1240, 2003