Obesity-prone rats have normal blood-brain barrier transport but defective central leptin signaling before obesity onset

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Obesity-prone rats have normal blood-brain barrier transport but defective central leptin signaling before obesity onset. Am J Physiol Regul Integr Comp Physiol 286: R143–R150, 2004. First published September 4, 2003; 10.1152/ajpregu.00393.2003.—Rats selectively bred to develop diet-induced obesity (DIO) were compared with those bred to be diet resistant (DR) on a 31% fat-high-energy diet with regard to their central leptin signaling and blood-brain barrier (BBB) transport. Peripheral leptin injection (15 mg/kg ip) into lean 4- to 5-wk-old rats produced 54% less anorexia in DIO than DR rats. DIO rats also had 21, 63, and 64% less leptin-induced immunoreactive phosphorylated signal transducer and activator of transcription 3 (pSTAT3) expression in the hypothalamic arcuate, ventromedial, and dorsomedial nuclei, respectively. However, hindbrain leptin-induced nucleus tractus solitarius pSTAT3 and generalized sympathetic (24-h urine norepinephrine) activation were comparable. Reduced central leptin signaling was not due to defective BBB transport since transport did not differ between lean 4- to 5-wk-old DIO and DR rats. Conversely, DIO leptin BBB transport was reduced when they became obese at 23 wk of age on low-fat chow or after 6 wk on high-energy diet. In addition, leptin receptor mRNA expression was 23% lower in the arcuate nuclei of 4- to 5-wk-old DIO compared with DR rats. Thus a preexisting reduction in hypothalamic but not brain stem leptin signaling might contribute to the development of DIO when dietary fat and caloric density are increased. Defects in leptin transport appear to be an acquired defect associated with the development of obesity and possibly age.

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pSTAT3 serves as a useful surrogate for the activation of OB-Rb (47, 61). In the present studies, we found that preobese, selectively bred DIO rats had reduced arcuate nucleus (Arc) OB-Rb expression in association with reduced leptin-induced anorexia and hypothalamic pSTAT3 expression at a time when leptin BBB transport was normal. Impaired leptin transport developed only after DIO rats became obese and/or aged.

METHODS

Animals and diet. Animal usage was in compliance with the Animal Care Committee of the East Orange and St. Louis Veterans Affairs Medical Centers (East Orange, NJ, St. Louis, MO) and the guidelines of the American Physiological Society (1). Male and female rats from our in-house colony of rats selectively bred to be DIO or DR were used at a variety of ages. These substrains were developed from the outbred Sprague-Dawley strain (Charles River Laboratories) and were bred for high (DIO) and low (DR) weight gain on a HE diet (33). Male and female rats from our parent colony in E. Orange (B. Levin) to the vivarium in St. Louis (W. A. Banks) where they were allowed to deliver and nurse their pups. These rats were kept at 22–23°C on a 12:12-h light-dark cycle (lights on at 0800) and were fed chow and water ad libitum from weaning. Leptin transport across the BBB was assessed in chow-fed male and female DR and DIO rats at 4 wk (n = 12 per group) and 11 wk of age (n = 8 per group). Transporter activity was also assessed in chow-fed male DR and DIO rats, which were either continued on Chow or switched to HE diet for 6 wk beginning at 11 wk of age (n = 8 per group). Transport activity was assessed in final groups of 12 DIO and 14 DR male rats, which were fed chow from weaning to 23 wk of age. Thus transport was assessed in Chow-fed DIO and DR males at 4, 11, 17, and 23 wk of age, as well as in males fed HE diet from 11 to 17 wk of age.

Assessment of leptin transport. Leptin (Amgen, Thousand Oaks, CA) was radioactively labeled by the lactoperoxidase method with 125I. Radiolabeled leptin (I-Lep) was separated from free iodine on a Sephadex G-10 column. The I-Lep had a specific activity of ~100–125 Ci/g and was stored at 4°C until use. The unidirectional influx rate of leptin from blood to brain was measured by multiple-time regression analysis (10, 50). Rats were anesthetized with pentobarbital, and the left jugular vein and right carotid artery were exposed. Rats were given an injection into the jugular vein of 0.2 ml of lactated Ringer solution containing 1% bovine serum albumin and about 5 x 10^5 counts/million (cpm) of I-Lep. Blood from the carotid artery was obtained between 1 and 10 min after intravenous injection (typically at 1-min intervals), centrifuged for 10 min at 5,000 g at 4°C, and the radioactivity level in the serum determined. Immediately after collection of each arterial blood sample, the rat was decapitated and the level of the radioactivity in the whole brain (with the pituitary and the pineal gland removed) determined. The brain-to-serum ratios of I-Lep in units of microliters per gram [(cpm/g of brain)/(cpm/μl serum)] were plotted against exposure time (Expt; in minutes)

\[
\text{Expt} = \frac{[\text{cP}]}{\text{Cp}(t)\text{dr}}/\text{Cp} \tag{1}
\]

where Cp is serum concentration, (t)dr is an integration time constant, and Cp is the level of radioactivity in serum at time t (10, 50). The slope of the linear portion of the relation between the brain-to-serum ratios and Expt was calculated by the least squares method (see Statistics below) and measures the unidirectional influx rate from blood to brain and is reported with its standard deviation of the residuals.
Selectively bred male DIO and DR rats (n = 8 per group) were placed on a reversed 12-h light-dark schedule during the first week of suckling, with lights off at 1200 (see Experiment I). They were fed Purina rat chow (no. 5001) ad libitum from weaning. At 5 wk of age, they were decapitated without fasting 2 h before lights on (1000–1200). Their brains were quickly removed and frozen on dry ice. At this time, trunk blood was taken for leptin assay by radioimmunoassay using antibodies directed against rat leptin, and so the reported values were multiplied by two to give “corrected rat” values.

Experiment III: OB-Rb expression. Selectively bred male DIO and DR (n = 8 per group) rats were placed on a reversed 12-h light-dark schedule during the first week of suckling, with lights off at 1200 (see Experiment I). They were fed Purina rat chow (no. 5001) ad libitum from weaning. At 5 wk of age, they were decapitated without fasting during the 2 h before lights on (1000–1200). Their brains were quickly removed and frozen on dry ice. At this time, trunk blood was taken for leptin assay by radioimmunoassay using antibodies directed against rat leptin (Linco). Brains were stored at –70°C until assay. A 300-μm section was cut from frozen brains on a cryostat at –12°C such that the compact portion of the DMN was in the center of the slice. Cut sections were placed in RNA Later (Ambion). After at least 1 h, sections were placed under a dissecting microscope, and the Arc, VMN, and DMN were micropunched bilaterally by minor modifications (30) of the method of Palkovits (49). The punched samples were kept in RNA for <2 wk before being reversed transcribed to cDNA.

Micropunch samples were subsequently assayed by real-time (quantitative) PCR. They were first homogenized ultrasonically in a solution containing guanidinium thiocyanate and nucleotides. Then mRNA was purified by using silica columns (Ambion RNAqueous kit). After removal of genomic DNA with DNase, mRNA was reversed transcribed with random hexamer priming using Superscript 3 (Invitrogen). Samples were then treated with RNaseH (Ambion), and the resulting purified cDNA was aliquoted and frozen. Primer sets for cyclophilin and OB-Rb mRNA were designed by reference to published sequences, and their specificity was verified by using Genbank and by comparing the sequenced PCR product for both cyclophilin and OB-Rb to these references. For each mRNA species, a pair of conventional primers was used in combination with a sequence-specific 6-carboxyfluorescein (FAM)-labeled probe to allow real-time PCR quantitation by using an Applied Biosystems 7700 Sequence detector set for 40 PCR cycles. The primers for cyclophilin were Genbank (NM 017101) forward (253–272) AATGGCACTGGTGGCAAGTC; reverse (330–310) GCCAGGACCTGTAGCCTCAG; FAM-labeled probe (275–290) TCTACGGAGAGAAATT. The primers for OB-Rb were Genbank (AF287268) forward (2913–2937) AACCTGTTGAGATGAGTGTGCAGAGTC; reverse (3005–2984) CCTGGCTCTTACATCAGTTTCCA; FAM-labeled probe ATGCAACGCTGGTCAGCAG (2954–2969). Reference standards for each hypothalamic nucleus were created for cyclophilin and OB-Rb from aliquots of samples from all samples of those areas used. These pooled samples were used to generate standard curves from which the respective quantitative data were read. Data were then expressed as the ratio of OB-Rb to cyclophilin.

Statistics. Food intake and urine NE levels after saline or leptin administration were assessed by one-way ANOVA for repeated measures with post hoc Bonferroni comparisons when significant differences were found. The number of cells expressing pSTAT3 and OB-Rb expression in each brain area were compared by two-way ANOVA (phenotype × treatment) with post hoc Bonferroni comparisons where appropriate.
Fig. 3. Effects of leptin on 24-h urine norepinephrine (NE) levels and food intake were assessed in 4- to 5-wk-old chow-fed male DR (n = 8; ○) and DIO (n = 9; ●) rats. A: 24-h urine NE levels (ng) at baseline (0) and after intraperitoneal administration of 10 and 15 mg/kg leptin. Data are means ± SE. *P ≤ 0.05 when leptin levels at a given dose were compared between DR and DIO rats. +P ≤ 0.05 when leptin levels for a given genotype after leptin were compared with their own baseline levels. B: leptin-induced (15 mg/kg ip) percent change in 24-h urine NE levels from baseline [Δ24-h urine NE (%Baseline)] compared with percent leptin-induced inhibition of 4-h food intake [intake inhibition (%Baseline)] in the same rat. There was a significant correlation for DR but not DIO rats.

For leptin transport, regression lines were calculated by the least squares method with the Prism 3.0 program (GraphPad, San Diego, CA) and the slope with its standard deviation of the mean reported. Regression lines were compared for statistical differences with the Prism 3.0 program, which first determines whether there are differences between slopes and, if not, whether there are differences between intercepts. When more than two slopes were compared, ANOVA was followed by Newman-Keuls range test with the standard deviation of the mean taken as the standard error term and, because two means (the slope and the intercept) were calculated from the data, n – 1 was used as the value for n.

RESULTS

Experiment I: leptin inhibition of food intake, stimulation of sympathetic activity, and pSTAT3 expression. Male 4- to 5-wk-old DIO and DR rats were injected with saline to determine baseline intake and then with leptin (10 and 15 mg/kg ip) to compare the effects on food intake, sympathetic activity (urine NE levels) and activation of the leptin signaling pathway (pSTAT3 expression). At 4 wk of age, DIO rats in this study weighed 85 ± 2 g and DR rats weighed 95 ± 3 g (P = 0.01). At 5 wk of age, DIO rats weighed 145 ± 4 g and DR rats weighed 152 ± 5 g (P > 0.1). Neither DIO nor DR rats had significant reductions in food intake at either 4 or 24 h after 10 mg/kg ip leptin compared with saline injections (data not shown). After 15 mg/kg ip leptin, 4-h intake was inhibited by 13% in DIO and 24% in DR rats compared with their salinetreated baselines (Fig. 1; P = 0.016). By 24 h, leptin inhibition of intake did not differ between DIO and DR rats (Fig. 1). After saline injection, there was little constitutive expression of pSTAT3 immunoreactivity in either hypothalamic or NTS neurons (Fig. 2). On the other hand, there was significant expression of pSTAT3 immunoreactivity in neurons within these areas after leptin injections. In the Arc, expression was most prominent in the medial portion (Fig. 2A). Overall, the number of Arc cells expressing pSTAT3 in DIO rats was only 79% of DR rats (Fig. 2B; P = 0.039). In the VMN, leptin induced a gradient of decreasing pSTAT3-expressing neurons from the dorsomedial to central to ventrolateral portions (Fig. 2A). In each portion of the VMN, DIO rats had only 36–38% as many pSTAT3-expressing neurons (Fig. 2B; P = 0.013 for VMN overall). In the DMN, most leptin-induced pSTAT3 expression occurred in the ventral portion (Fig. 2A), and DIO rats had only 38% as many pSTAT3 immunoreactive DMN cells as DR rats (Fig. 2B). Finally, there was moderate expression of leptin-induced pSTAT3 in the NTS. However, in contrast to hypothalamic leptin-induced pSTAT3 induction, there was no significant difference between DIO and DR rats in pSTAT3 induction in the NTS (Fig. 2B).

At baseline, 4-wk-old DIO rats had 35% higher 24-h urine NE levels than DR rats (Fig. 3A; P = 0.05). As with its effect on food intake, injection of 10 mg/kg ip leptin did not alter 24-h urine NE levels in either genotype, although DIO rats no longer had higher levels than DR rats. After 15 mg/kg leptin, 24-h urine NE levels rose significantly above baseline in both DIO (127%; P = 0.006) and DR rats (110%; P = 0.05), and DIO levels were again higher (46%; P = 0.01) than DR levels. When the leptin-induced percent changes from baseline for 24-h urine NE and 4-h intake inhibition were plotted against each other (Fig. 3B), there was a significant correlation between these parameters in DR (r = 0.90; P = 0.01) but not in DIO rats. Curiously, half of the DR rats actually had a reduced 24-h urine NE response to leptin administration compared with saline-injected controls (Fig. 3B). There were no correlations between leptin-inhibition-induced of 24-h intake and 24-h urine NE levels for either genotype.

Experiment II: Effects of genotype, gender, diet, and age on BBB leptin transport. The impaired anorectic and hypothalamic pSTAT3 expression in 4- to 5-wk-old DIO rats might have been due to reduced BBB transport. To address this issue, transport was compared between DIO and DR rats of differing ages, sexes, and degrees of obesity. At 4 wk of age, there were no differences in leptin transport across the BBB among lean, chow-fed DR or DIO males or females. All four groups had

| Table 1. Transport rates across the BBB (Ki) of I-Lep and body weights in 4-wk-old DR and DIO rats |
|----------------|----------------|--|
| Group         | Ki, µl/g-min   | Body Weight, g |
|               |                |                |
| DR male       | 0.731 ± 0.124  | 90 ± 4*        |
| DIO male      | 0.768 ± 0.163  | 80 ± 4†        |
| DR female     | 0.687 ± 0.155  | 86 ± 5‡        |
| DIO female    | 0.723 ± 0.139  | 75 ± 5§        |

Values are means ± SE. Leptin was transported from blood to brain in all groups. BBB, blood-brain barrier; Ki, unidirectional influx rate; DR, diet resistant; DIO, diet-induced obesity. **§Significantly different from each other at P = 0.05 or less by post hoc analysis.
similar transport rates (Table 1; Fig. 4). Body weights differed among the four groups, and the weights of each group were different from those of the other three [Table 1: F(3,43) = 24.5; P < 0.001]. At this age, as in the 4-wk-old rats in Experiment I, DIO rats weighed 10% less than their DR counterparts. At 11 wk of age, there were further differences in body weight among the groups [Fig. 5: F(3,28) = 238; P < 0.001]. DIO males weighed ~29% more than DR males (P < 0.001), and DIO females weighed ~26% more than DR females (P < 0.001). The mean serum leptin level in seven DIO males (2.54 ± 0.31 ng/ml) was twice as high as in seven DR males (1.34 ± 0.39 ng/ml; P < 0.05). However, there were no significant differences in transport rate among the four groups (Fig. 5).

When 11-wk-old male rats were either switched to HE diet or continued on chow for 6 wk, there were further significant intergroup differences in body weight [Fig. 6; F(3,28) = 2.5; P < 0.001]. DIO rats fed HE diet weighed 24% more than chow-fed DIO rats, 43% more than DR rats fed HE diet, and 54% more than chow-fed DR rats (P < 0.001 for all). The 7% difference in body weight between chow-fed DR and DR rats fed HE diet was not statistically different. The chow-fed DR and DIO rats and DR rats fed HE diet all transported I-Lep into the brain at similar rates of transport that did not differ from one another. However, only small amounts of labeled leptin were recovered from the brains of DIO rats fed HE diet and the ratios of brain to serum leptin were extremely variable among the animals. Thus the relationship between brain-to-serum ratios and time was not statistically significant for this group, i.e., no statistically significant transport had occurred in DIO rats fed HE diet. Nevertheless, a regression line was calculated for these animals to allow for intergroup statistical comparison. This comparison showed significant differences among the slopes of the regression lines for the four groups [F(3,23) = 4.12; P < 0.05]. Post hoc analysis showed that the slope of the line for DIO rats on HE diet was different from that of DR rats on HE diet (P < 0.05) and from that of chow-fed DIO rats (P < 0.05). The half-time disappearance rate of I-Lep from blood did not differ among these four groups, demonstrating that this saturable clearance mechanism was not affected.

Final groups of DR and DIO rats were fed chow from weaning to 23 wk of age and were then evaluated for leptin transport (Fig. 7). Diet had no effect on transport rates in DR rats at this age, but the DIO group had lost the ability to transport leptin and was, therefore, significantly lower than time-matched chow-fed DR rats (P < 0.05). Figure 7 summarizes the changes in leptin transport rates for chow-fed DR and DIO male rats at 4, 11, 17, and 23 wk of age.

**Experiment III: DIO and DR OB-Rb mRNA expression.** At 5 wk of age, when peripheral leptin levels were statistically comparable in chow-fed DIO (2.3 ± 0.2 ng/ml) and DR rats (1.8 ± 0.2 ng/ml), DIO Arc OB-Rb mRNA expression was 77% of that in DR rats (Table 2). Despite reduced leptin-induced pSTAT3 expression in both the VMN and DMN of DIO rats, there were no differences in OB-Rb expression between DIO and DR rats in those hypothalamic nuclei (Table 2).

**DISCUSSION**

The rat model of DIO is a good surrogate for human obesity. As with human obesity (11), the DIO trait appears to be inherited in a polygenic fashion (33, 34), and DIO rats develop many features of the metabolic syndrome (19, 41, 42, 60, 63). In this study, we show that selectively bred DIO rats have a defect in central leptin signaling, which antedates the onset of obesity. At 4–5 wk of age, selectively bred DIO rats had reduced Arc OB-Rb expression in association with an attenuated anorectic response and activation of hypothalamic pSTAT3 after peripherally administered leptin. This defect was not attributable to deficient BBB transport of leptin since transport did not differ between DIO and DR rats at this age.
DIO rats acquired defective leptin transport only as they aged and/or became obese. Their reduced OB-Rb mRNA and leptin-induced pSTAT3 expression in the Arc, a nucleus known for its critical role in energy homeostasis (40, 57), suggests that impaired Arc leptin signaling might underlie their impaired anorectic response to leptin. On the other hand, the lack of impaired leptin-induced activation of pSTAT3 in the NTS, increased leptin-induced urine NE levels, and their known propensity to develop obesity-related hypertension (19) suggest that leptin signaling in this critical hindbrain autonomic nucleus (44) might not be altered in DIO rats.

The reduced Arc OB-Rb mRNA expression in DIO rats exists before the development of obesity or exposure to HE diet. Reduced mRNA expression does not guarantee that receptor protein expression or binding will be decreased. However, their attenuated leptin-induced Arc pSTAT3 expression suggests that DIO rats had a parallel or even greater reduction in functional Arc leptin signaling (21, 23, 27, 28, 47). It is unclear why there were no similar reductions in DIO VMN and DMN OB-Rb expression by quantitative PCR since their leptin-induced pSTAT3 expression was reduced. Also, our laboratory previously found (35) reduced OB-Rb mRNA expression in both the VMN and DMN of DIO rats by in situ hybridization. However, those reductions were small (~10%) and might not have been detected here by the combination of micropunch and quantitative PCR. Although there is no conclusive evidence that leptin might affect food intake by a direct effect on the VMN, there is evidence that the DMN might play a role comparable to that ascribed to the Arc (8) and that this might be mediated, in part, by leptin (9). However, the selective decrease of both OB-Rb expression and leptin-induced STAT3 activation in the Arc of DIO rats, along with the well-established role of the Arc in energy homeostasis (40, 57), suggests that defective Arc leptin signaling might be a critical determinant of the development of obesity in DIO rats fed HE diet. One cautionary note is that the leptin signaling pathways involved in producing anorexia might not be dependent on STAT3 activation. But assessment of immunoreactive pSTAT3 activation is currently the best surrogate for short-term postreceptor signaling available at the cellular level for leptin signaling.

Although the present studies suggest that 4- to 5-wk-old DIO rats have reduced leptin sensitivity compared with DR rats, they actually had lighter body weights at 4 wk of age. Because rats at this age have only 5–6% body fat (52), it is likely that their differences in body weight were due to relatively less lean body mass in DIO rats. Although there is no obvious explanation for this apparent reversal of expected relative body weights, it is unlikely that it is due to the lighting schedule used. DIO rats raised under both reversed (Experiment I) and conventional lighting schedules (Experiment II) weighed less than DR rats at 4 wk of age. Although it is unclear why there were differences in body weight at 4 wk of age, these differences disappeared at 5 wk of age.

The reduced responsiveness of 4- to 5-wk-old DIO rats to peripherally injected leptin cannot be explained by reduced access of leptin to central sites. DIO rats at this age had comparable BBB leptin transport to DR rats. Also, outbred DIO rats, from which the selectively bred DIO rats in the present studies are derived (33), have reduced sensitivity to the

Table 2. Real-time PCR ratio of hypothalamic leptin receptor to cyclophilin mRNA

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<th>DR</th>
<th>DIO</th>
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<tr>
<td>Arc</td>
<td>1.18±0.05</td>
<td>0.91±0.09*</td>
</tr>
<tr>
<td>VMN</td>
<td>1.09±0.07</td>
<td>1.12±0.08</td>
</tr>
<tr>
<td>DMN</td>
<td>1.07±0.12</td>
<td>1.14±0.14</td>
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Values are means ± SE. Chow-fed male 5-wk-old selectively bred DIO and DR rats (8 per genotype) had their hypothalamic arcuate (Arc), ventromedial (VMN), and dorsomedial (DMN) nuclei micropunched and subjected to real-time PCR. *P = 0.05 when DIO were compared with DR rats.
anorectic effects of centrally administered leptin (32). Moreover, no BBB transport would be required for leptin to reach some neurons in the Arc or NTS since both reside close to brain areas with no BBB (13, 22). Because a major portion of leptin’s anorectic effect is mediated through Arc neurons (48, 57), peripherally administered leptin might reach this target, engage the OB-Rb on Arc neurons and activate STAT3, as well as producing anorexia without having to cross the BBB. Similarly, the fact that transport should not be required for STAT3 activation in the NTS suggests that differences between DIO and DR rats in the anorectic effects of peripherally administered leptin are not mediated through the NTS because there was no difference in leptin-induced pSTAT3 activation in this nucleus.

The dissociation between the anorectic effect of leptin and 24-h urine NE levels in DIO rats is in agreement with other studies showing a dissociation between the anorectic and sympathoexcitatory effects of leptin (17, 46, 52). In fact, this dissociation might explain why DIO rats have constitutively elevated urine NE levels (29, 30, 33) and develop obesity-associated hypertension (19) but do not downregulate their food intake or Arc neuropeptide Y expression until plasma leptin levels appreciably exceed those of DR levels (32, 37). Aside from its generally catabolic effect on energy homeostasis, leptin increases both general sympathetic activity and blood pressure (18, 26). Interestingly, the dissociation between leptin-induced anorexia and sympathoexcitation did not hold for DR rats, possibly because urinary NE was used as an index of sympathetic activity. Clearly, sympathetic activity varies by organ system. For example, although DIO-prone rats have constitutively elevated levels of urine NE excretion (29, 33), they actually have reduced cardiac and pancreatic, but not brown adipose, sympathetic activity (30). Similarly, the effect of leptin on sympathetic activity appears to be highly organ specific (25, 45). Another possibility is that altered urine NE levels might be due to an effect of leptin on the renal clearance of NE (16).

Leptin sensitivity appears to decrease as a function of age, increasing dietary fat content and obesity (3, 15, 21, 43, 54–56, 62, 64). In rats, these factors are generally not dissociable, since most rat strains become obese with age and on high-fat diets. Here, selectively bred DIO rats developed defective leptin transport only when fed HE diet for 6 wk from 11 wk of age or when fed chow from weaning to an age of 23 wk. Although we did not measure leptin levels or carcass fat in the DIO and only sporadically available. When food was readily available, rats could store more energy as fat before leptin exerted its inhibitory effect on intake and stimulatory effects on thermogenesis. In humans, reduced leptin signaling should confer a similar competitive advantage where energy sources were limited. However, in times of excess food availability, it would promote the development and maintenance of obesity.

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