20-Hydroxyecdysone decreases weight and hyperglycemia in a diet-induced obesity mice model

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ECDYSTEROIDS ARE POLYHYDROXYLATED STEROIDS present in plants and invertebrates. So far, close to 120 ecdysteroids have been structurally characterized (18). The chemical structure of ecdysteroids is based on the C-27 cholesterol skeleton; however, they differ from the vertebrate steroids in their polarity and biochemical properties. In mammals, the median lethal dose (LD50) of 20HE in rats is 6.4 g/kg body wt when given orally (25). Moreover, ecdysteroid-rich extract from Ajuga turkestana administered orally (5 mg/kg) was more effective than the reference drug manilil in reducing blood glucose in a normal diabetic mice model (26).

An important aspect of the ecdysteroids is their toxicity to invertebrates. The median lethal dose (LD50) of 20HE in invertebrates such as Drosophila, Caenorhabditis elegans, and Artemia franciscana is 20-40 mg/L (27). However, the ecdysteroids are not toxic to mammals and have been shown to be safe at doses up to 500 mg/kg body wt (28).

MATERIALS AND METHODS

Chemicals. Ecdysterone was purchased from Bosche Scientific (New Brunswick, NJ). Dexamethasone, 8-(4-chlorophenylthio)-cAMP (CAMP), sodium lactate, and sodium pyruvate were purchased from Sigma Chemicals (St. Louis, MO). Human insulin (Humulin) was purchased from Eli Lilly (Indianapolis, IN) and compound C from Sigma Chemicals (St. Louis, MO). Human insulin (Humulin) was purchased from Eli Lilly (Indianapolis, IN) and compound C from EMD Biosciences (San Diego, CA); phospho-Akt1 and Akt2 rabbit mAbs were purchased from Cell Signaling Technology (Danvers, MA). All other chemicals, including cell culture media, were obtained from Invitrogen (Carlsbad, CA). Reagents and enzymes used for RT-PCR were obtained from Stratagene (La Jolla, CA) and Applied Biosystems (Foster City, CA).

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BioSystems (Foster City, CA). The H4IE cell line (CRL-1548) was obtained from American Type Culture Collection (Manassas, VA).

**Cell culture and treatment.** The H4IE hepatoma cells were cultured in 24-well tissue culture plates (Greiner Bio One, Monroe, NC) and grown to near confluence in Dulbecco’s modified Eagle’s medium containing 2.5% (vol/vol) newborn calf serum and 2.5% (vol/vol) fetal calf serum. Cells were treated for 8 h with 500 nM dexamethasone and 0.1 mM 8-CTP-cAMP (Dex-cAMP) to induce phosphorylated eukaryotic translation initiation factor-2 (eIF-2α) and glucose-6-phosphatase (G6Pase) gene expression together with different concentrations of 20HE, or 10 nM insulin. Three wells were allocated for each treatment, including the negative control (untreated cells). For inhibitory assays, cells were pre-treated for 30 min with either 20 μM LY-294002 or 40 μM compound C as specified, washed with phosphate-buffered saline, and incubated for an additional 7 h with Dex-cAMP together with different concentrations of 20HE, or 10 nM insulin.

**Glucose production assay.** H4IE rat hepatoma cells were serum starved overnight in the glucose production buffer (glucose-free Dulbecco’s modified essential medium, pH 7.4, containing 20 mM sodium lactate and 2 mM sodium pyruvate without phenol red) and treated for 8 h with Dex-cAMP in the presence or absence of 10 nM insulin or different concentrations of 20HE for 8 h. At the end of the incubation, 0.5 ml of medium was taken to measure the glucose concentration in the culture medium using the Amplex Red glucose assay kit (Invitrogen). Corrections for cell number were made on the basis of the protein concentration measured using the BCA Protein assay kit (Pierce Biotechnology, Rockford, IL).

Total RNA extraction, purification, and cDNA synthesis. Total RNA was extracted from H4IE cells or liquid nitrogen-preserved murine tissues using Trizol reagent (Invitrogen) following the manufacturer’s instructions. RNA was quantified spectrophotometrically by NanoDrop Technologies, Wilmington, DE. Quality of RNA was assessed by separation in gel electrophoresis. To remove any traces of DNA contamination, RNA was then treated with DNase I (Invitrogen) following the manufacturer’s guidelines. The cDNAs were synthesized using 2.5 μg of RNA for each sample, using StrataScript Reverse Transcriptase (Stratagene) following the manufacturer’s protocol.

**Quantitative PCR analysis of H4IE rat hepatoma cells.** The synthesized cDNAs were diluted fourfold. Five microliters of each diluted sample were used for a PCR reaction of 25 μl final volume containing 0.5 μl of 6 μM gene-specific primers (IDT, Coralville, IA) and 12.5 μl of Brilliant SYBR green PCR master mix (Stratagene). The Dex-cAMP or treatment (positive control) served as the calibrator sample in this study, and the target gene expression of the calibrator sample was assigned to a value of 1.0. All samples were run in triplicate. A P value of 0.05 was considered to be significant.

**AMPKα1 and α2 activity assay.** AMPK activity was assayed as previously described (12). Briefly, AMPK was immunoprecipitated from 200 μg of H4IE cell lysate using anti-AMPKα1 (Upstate Biotechnology, Lake Placid, NY) or α2 (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies in 500 μl of buffer A (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM benzamidem, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin) at 4°C for 2 h. Immunocomplexes were washed with buffer A three times, buffer B containing 5 mM NaCl and 62.5 mM NaF one time, and then the reaction buffer (50 mM HEPES, pH 7.4, 1 mM DTT) three times. AMPK activity of immunocomplexes was determined by phosphorylation of SAMS peptide in the reaction buffer containing 0.25 mM SAMS, 5 mM MgCl2, and 10 μg of [γ-32P]ATP at 30 min at 30°C with or without 200 μM AMP stimulation. The reaction was terminated by spotting reaction mixtures onto P81 filter paper and rinsing in 1% (vol/vol) phosphoric acid with gentle stirring to remove free ATP. The phosphorylated substrate was measured by scintillation counting.

**Western blot analysis.** H4IE cells were cultured as described above, and whole cell extracts were prepared in ice-cold lysis buffer [62.5 mM Tris-HCl (pH 6.8), 2% wt/vol SDS, 10% glycerol, 50 mM DTT, 0.01% vol/vol bromophen blue] and centrifuged at 12,000 g for 20 min at 4°C. Equal amounts of protein (50 μg) from the supernatants were separated on SDS 10% polyacrylamide gels and blotted onto the nitrocellulose membrane. Western blot analysis was performed with monoclonal phospho-Akt (Ser473) antibodies according to the manufacturer’s instructions (Cell Signaling Technology, Danvers, MA). After being washed, the blots were incubated with an anti-rabbit peroxidase-labeled secondary antibody and visualized using ECL Western Blotting Detection Reagent (GE Healthcare, Piscataway, NJ). After being stripped, the blots were probed with Akt2 (BS5) antibodies to visualize the total Akt (loading control).

**Animal experiments.** All animal experiments were performed according to procedures approved by the Rutgers Institutional Animal Care and Use Committee. Six-week-old male C57BL/6J mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and maintained on either a low-fat diet (LFD; n = 10) containing 10% fat-derived calories (D12450B; Research Diets, New Brunswick, NJ) or an HFD (n = 10) containing 35% fat-derived calories (TD88137; Research Diets, New Brunswick, NJ) with 50% sucrose at 0.5% in the diet, as described previously (26).
containing 60% fat-derived calories (D12492, Research Diets) with 12-h light and dark cycles.

The HFD animals were further randomized into two groups. The control group (n = 10) was gavaged daily with a vehicle solution alone (10% DMSO in corn oil), and a treatment group (n = 10) was gavaged with 10 mg/kg body wt 20HE for 13 wk. To monitor gain and loss of body weight, the animals were weighed weekly for the duration of the experiment. The mice in the intraperitoneal treatment was monitored using a thermometor containing a probe (Oakton Instruments, Vernon Hills, IL). Plasma glucose concentrations were measured at weeks 4, 9, 10, 11, and 12 in submandibular vein blood samples using a glucometer (LifeScan, Johnson and Johnson, NJ). Plasma concentrations of insulin and adiponectin were determined at week 13 by rat/mouse insulin ELISA kit (Millipore, Billerica, MA) and adiponectin ELISA kit (Otsuka Pharmaceuticals, Toyko, Japan), respectively.

To perform the glucose tolerance test at week 13 of the experiment, both LFD and HFD mice were fasted overnight (16 h) and injected intraperitoneally with 1.5 g/kg glucose solution. Plasma glucose levels were measured immediately before and 30, 60, and 120 min after the glucose challenge. At the end of the study, mice were killed and equal amounts of liver and visceral fat were removed. The fat mass and lean tissue were determined using dual-energy X-ray absorptiometry (DEXA) analysis on PIXimus equipment (Lunar, Madison, WI) as described elsewhere (27). The percentage of fat tissue was calculated as follows: %body fat = (fat mass/total body wt) × 100, where total body weight was the sum of lean mass and fat mass for each animal. The ratio between adipose mass and lean mass for each animal was calculated by dividing fat mass by lean mass.

RESULTS

20HE modulates glucose metabolism in rat hepatoma cells. The production of glucose in response to 20HE was tested in H4IIE rat hepatoma cells incubated in medium containing pyruvate and lactate as substrates for gluconeogenesis. The cells were serum starved for 16 h and incubated with different concentrations of 20HE for 8 h with or without insulin. Absolute glucose production numbers were normalized to the total cell protein and transformed to represent relative glucose production units as percentage of Dex-cAMP-stimulated glucose production value (set as 100% for each experiment). 20HE decreased the glucose production in a dose-dependent manner with 20HE at 10 μM showing similar inhibition as insulin at physiological concentration (10 nM). The inhibitory effect of 20HE over glucose production was mediated by the activation of PI3K and could be reversed by specific PI3K inhibitor LY-294002 (Fig. 1A). A quantitative analysis of the mRNA expression patterns of PEPCK and G6Pase in Dex-cAMP-stimulated and 20HE-treated H4IIE cells was performed to determine whether the effect of 20HE on glucose production is related to its effect on PEPCK or G6Pase, two of the rate-limiting enzymes regulated by insulin in the hepatic gluconeogenic pathway. 20HE significantly decreased PEPCK and G6Pase gene expression in Dex-cAMP-induced cells in a dose-dependent manner (Fig. 1, B and C). Furthermore, the inhibitory effect of 20HE on the expression of both gluconeogenic enzymes was mediated by the activation of PI3K and could be reversed by specific PI3K inhibitor LY-294002 (Fig. 1, B and C). Untreated cells were used to measure the basal level of PEPCK and G6Pase expression, while the β-actin gene was chosen as an internal standard since the level of β-actin mRNA remained unaffected by the treatments. Moreover, 20HE at concentrations of 2.5 and 10 μM induced Akt2 phosphorylation within the carboxy terminus at Ser473. This effect was reversed by the PI3K inhibitor LY-294002 (Fig. 1D). Compound C, an ATP-competitive inhibitor of AMPK (26), did not reverse the effects of 20HE on Dex-cAMP-stimulated PEPCK gene expression in H4IIE cells (data not shown).

20HE activates AMPKα1 in rat hepatoma cells. Liver AMPK is another PI3K-independent signaling pathway that decreases expression of glycolytic and lipogenic genes, as well as genes involved in hepatic glucose production (37). The effects of 20HE on basal and AMP-stimulated activity of AMPKα1 catalytic subunit in H4IIE rat hepatoma cells are shown in Fig. 2. AMP levels were significantly higher in cells treated with different concentrations of 20HE compared with control levels. Indeed, 20HE at 2.5, 10, and 50 μM increased AMPKα1 activation by 4, 14, and 32%, respectively, over the control values. On the other hand, AMPKα2 catalytic subunit activity was not affected by the 20HE treatment (data not shown).

Because AMPKα2 is the main AMPK catalytic subunit expressed in muscle and liver, it is likely that a moderate AMPK-mediated effect of 20HE on glucose metabolism is secondary to activation of the PI3K signaling pathway. Therefore, we focused on the PI3K-related mechanism on 20HE action in the subsequent animal studies.

20HE-treated mice resist diet-induced obesity. Obesity was induced by feeding mice for 13 wk with the HFD. The final body weights were 41.7 ± 1.66 vs. 26.7 ± 0.74 g for HFD and LFD animals, respectively. In the HFD animals receiving a daily oral gavage of 10 mg/kg 20HE, final body weights were markedly decreased (36.5 ± 1.56 g, P < 0.05 compared with HFD animals) (Fig. 3). Body weight gain relative to the initial body weight in mice treated with 20HE was 18% less compared with the HFD control (15.4 ± 0.5 vs. 18.8 ± 0.57 g, P < 0.05), while the body weight gain of the LFD mice remained low (5.18 ± 0.25 g). Reduction in body weight and body weight gain in the 20HE-treated group could not be attributed to changes in animal feeding habits, since food consumption rates remained unchanged throughout the treatment (2.2 ± 0.02 g/day for all groups). DEXA analysis (4) indicated that adipose mass in the 20HE-treated mice was decreased by 41% when compared with the HFD animals (13.1 ± 0.67 vs. 22.3 ± 1.0 g, P < 0.05), while lean mass was decreased by only 5% (21.3 ± 0.09 vs. 22.3 ± 0.72 g). The ratio between adipose mass and lean mass of the 20HE-treated animals was significantly reduced relative to that of the HFD animals (0.62 ± 0.05 vs. 1.0 ± 0.05, P < 0.05, respectively). For the LFD-fed animals, this ratio was 0.25 ± 0.07. Thus the augmentation in body weight gain in the 20HE-treated mice was predominantly due to decreased adipose mass and enhanced resistance to diet-induced obesity. There was no detectable change in body temperature in either the HFD or the HFD+20HE animals when measured at the end of the experiment.

Adiponectin expression and production are enhanced by 20HE treatment. Adiponectin is involved in glucose and lipid metabolism, increases fatty acid oxidation in the muscle, and potentiates insulin inhibition of hepatic gluconeogenesis (36), and the circulating levels of adiponectin are reduced in obese subjects (33). Adiponectin mRNA was quantified in the adipose tissue of the LFD, HFD, and HFD-fed 20HE-treated animals. Thirteen weeks of daily oral treatment of HFD-fed C57BL/6J mice with 10 mg/kg 20HE resulted in a 7.9-fold
increase in visceral fat adiponectin expression compared with the control animals fed an HFD with a fold ratio equal to 1. The LFD-fed animals displayed a 5.2-fold increase in adiponectin expression in visceral fat compared with the HFD-fed animals. Plasma adiponectin levels measured at the end of the study (week 13) did not change among the tested groups (data not shown); however, when plasma adiponectin levels were adjusted using either fat mass weight or body weight, a significant increase was found in the mice treated with 20HE (Fig. 4, A and B, and Table 1).

Effect of 20HE on glucose metabolism and plasma insulin. The baseline blood glucose levels did not differ in all groups 4 wk after the start of treatment (Fig. 5A). Starting on week 9, the blood glucose of the HFD group increased steadily while the 20HE-treated group maintained a constant level of blood glucose, similar to that of the LFD group. Table 1 shows that plasma insulin levels measured at the end of the study were significantly higher in the HFD animals compared with the LFD group. 20HE treatment resulted in a 4.5-fold decrease in plasma insulin compared with the HFD group and a 1.7-fold decrease compared with the basal insulin levels observed in the LFD animals. An intraperitoneal glucose tolerance test was performed in week 13 of the experiment. The HFD group plasma glucose levels were significantly higher at 30, 60, and 120 min after oral gavage compared with the LFD group, while the 20HE treatment significantly reversed this effect at 30 and 60 min (Fig. 5B). Expression of PEPCK and G6Pase gluconeogenic enzymes was determined in the liver tissue at the end of the study. In animals treated with 20HE, the RNA levels were significantly reduced for PEPCK and G6Pase, respectively (Fig. 5C).

To determine whether 20HE affects hepatic lipogenesis, we analyzed the pattern of expression of SREBP-1 in liver tissue; however, we did not notice any significant change in SREBP-1.
mRNA expression in animals fed the HFD when compared with animals fed HFD and treated with 20HE (data not shown).

**DISCUSSION**

Earlier data suggest that 20HE has hypoglycemic effects both in vitro and in vivo (6, 38); however, no mechanism explaining this pharmacological activity had been proposed. Present studies have begun to reveal the anti-diabetic mode of action of 20HE in H4IIE rat hepatoma cell line and in a murine model of diet-induced obesity. Our study confirms that 20HE reduces glucose production in the hepatic cell culture (Fig. 1A) and suggests that this effect is regulated predominantly through PI3K-dependent signaling pathways. 20HE downregulated expression of PEPCK and G6Pase genes in a dose-dependent manner (Fig. 1, B and C). PEPCK is a key enzyme modulating hepatic gluconeogenesis, and its activity is closely correlated with hepatic glucose output (13), while G6Pase catalyzes the last step of hepatic glucose production by hydrolyzing glucose-6-phosphate into glucose (35). LY-294002 is a specific inhibitor of the PI3K pathway (31) known to decrease insulin output by affecting the upstream components of the PI3K-dependent insulin signaling pathway. Indeed, LY-294002 significantly inhibits Akt2 phosphorylation induced by 20HE or insulin (Fig. 1D). The dose response of 20HE for glucose secretion appears biphasic with a significant effect at 0.1 μM (Fig. 1A), while no effect on PEPCK or G6Pase gene expression is observed at 2.5 μM (Fig. 1, B and C). It is therefore possible that at lower concentrations, there is an unknown non-PEPCK/G6Pase-mediated effect of 20HE on glucose output that remains LY-294002 sensitive.

The biguanidine drugs of plant origin, such as metformin, exert a PI3K-independent downregulation of basal PEPCK gene transcription in hepatoma cells (1). The inhibitory effect of 20HE on glucose production and expression of gluconeogenic enzymes was abolished in the presence of this specific PI3K inhibitor (Fig. 1, A–C), suggesting that 20HE suppresses glucose output by affecting the upstream components of the PI3K-dependent insulin signaling pathway. Indeed, LY-294002 significantly inhibits Akt2 phosphorylation induced by 20HE or insulin (Fig. 1D). The dose response of 20HE for glucose secretion appears biphasic with a significant effect at 0.1 μM (Fig. 1A), while no effect on PEPCK or G6Pase gene expression is observed at 2.5 μM (Fig. 1, B and C). It is therefore possible that at lower concentrations, there is an unknown non-PEPCK/G6Pase-mediated effect of 20HE on glucose output that remains LY-294002 sensitive.

The biguanidine drugs of plant origin, such as metformin, exert a PI3K-independent downregulation of basal PEPCK gene expression in hepatocytes (39) through an AMPK-dependent mechanism. This pathway leads to the insulin-independent suppression of hepatic gluconeogenesis by phosphoryla-

**Fig. 2.** Effect of different concentrations of 20HE on basal and AMP-stimulated activity of the AMP-activated protein kinase (AMPKα1 catalytic subunit in H4IIE rat hepatoma cells. Cells were incubated with 0.1% DMSO (vehicle) and 2.5, 10, and 50 μM 20HE. The percentage of increase of AMPKα1 activation was 4, 14, and 32% for 2.5, 10, and 50 μM 20HE, respectively. Data represent the mean of 3 experiments ± SE. **P < 0.05, ***P < 0.01. Student’s t-test comparison for 20HE treatments vs. control.

**Fig. 3.** Effect of 20HE on the relative body weight gain of C57BL/6J mice. Six-week-old male mice were fed a low-fat diet (LFD), high-fat diet (HFD), or HFD combined with daily gavage with 10 mg/kg 20HE (HFD+20HE) for 13 wk. *P < 0.05, significantly different from HFD-fed vehicle-treated animal values. One-way ANOVA, Dunn’s post hoc test. This study is representative of 2 independent experiments.

**Fig. 4.** 20HE increases relative circulating adiponectin levels in plasma. Circulating adiponectin levels were measured by ELISA and normalized to fat mass (A) or body weight (B) of the animals. Values are means ± SE. *P < 0.05, Student’s t-test comparison for 20HE treatment vs. HFD control. This study is representative of 2 independent experiments.

**Table 1.** Effect of daily oral treatment with 10 mg/kg 20HE on C57BL/6J mice after 13 wk on plasma insulin and adiponectin levels

<table>
<thead>
<tr>
<th>Group</th>
<th>Insulin, ng/ml</th>
<th>Plasma Adiponectin/Body Fat</th>
<th>Plasma Adiponectin/Body Weight</th>
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<tr>
<td>LFD</td>
<td>0.63±0.03</td>
<td>934.3±33</td>
<td>4,872±200</td>
</tr>
<tr>
<td>HFD</td>
<td>1.74±0.14</td>
<td>715.6±36</td>
<td>1,441±73</td>
</tr>
<tr>
<td>HFD+20HE</td>
<td>0.38±0.03*</td>
<td>978.3±30.2*</td>
<td>2,500±16*</td>
</tr>
</tbody>
</table>

Data represent the mean of 2 different experiments ± SE. Insulin concentration is expressed as ng/ml, and adiponectin concentrations are ng·μl−1·g body fat−1 and ng·μl−1·g body wt−1. LFD, low-fat diet; HFD, high-fat diet; 20HE, 20-hydroxyecdysone. *P < 0.05, Student’s t-test comparison for 20HE treatments vs. HFD control.

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pressed AMPK1 catalytic subunit, but had no effect on rapamycin complex-2 transcriptional coactivator (17). 20HE and cytoplasmic sequestration of the mammalian target of energy expenditure and fatty acid oxidation in liver and skeletal muscles (36), one could postulate that relative adiponectin levels are elevated in 20HE-treated mice might confer resistance to diet-induced obesity and insulin resistance in these animals. Additionally, circulating adiponectin is known to suppress the expression of adiponectin mRNA expression observed in the HFD animals was completely reversed by 20HE treatment. Although the treatment did not lead to a marked increase in absolute plasma adiponectin levels, the relative circulating adiponectin increased significantly when adjusted to fat mass or body weight (Fig. 4, A and B). There is an inverse correlation between plasma adiponectin levels and body weight resulting from downregulation of the expression of adiponectin within adipose tissue as fat mass increases (2). Reduced adiponectin mRNA expression observed in HFD animals was completely reversed by 20HE treatment. To understand the anti-obesity effects of 20HE, we focused on adiponectin, an adipose tissue-specific secretory plasma protein with well-established effects in the regulation of insulin resistance, energy homeostasis, and obesity (3, 5, 24). Plasma concentrations of adiponectin are reduced in obese, insulin-resistant, and type 2 diabetic humans and rodents (15, 16, 33). In the present study, reduced adiponectin expression observed in HFD animals was completely reversed by 20HE treatment. Although the treatment did not lead to a marked increase in absolute plasma adiponectin levels, the relative circulating adiponectin increased significantly when adjusted to fat mass or body weight (Fig. 4, A and B). There is an inverse correlation between plasma adiponectin levels and body weight resulting from downregulation of the expression of adiponectin within adipose tissue as fat mass increases (2). Reduced adiponectin mRNA expression observed in HFD animals compared with the LFD controls was completely reversed by 20HE treatment. 20HE significantly increased circulating adiponectin levels relative to fat mass or body weight of the treated animals (Fig. 4, A and B). Because adiponectin increases energy expenditure and fatty acid oxidation in liver and skeletal muscles (36), one could postulate that relative adiponectin levels elevated in 20HE-treated mice might confer resistance to diet-induced obesity and insulin resistance in these animals. Additionally, circulating adiponectin is known to suppress the expression of PEPCK and G6Pase genes by the activation of the AMPK signaling pathway (36).
In conclusion, our results shed new light on the hypoglycemic effect of 20HE in vitro and in vivo and indicate that this effect may be exerted through the PI3K-dependent regulation of gluconeogenic enzyme activity. Even more important, the data suggest that daily administration of 20HE can prevent obesity, insulin resistance, and associated hyperglycemia in animals by decreasing adipose depots, upregulating adiponectin expression in the adipose tissue, and increasing circulating adiponectin levels adjusted to body weight or fat mass.

GRANTS

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