Regulation of basal, pulsatile, and entropic (patterned) modes of GH secretion in a putatively low-somatostatin milieu in women

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Submitted 2 March 2009; accepted in final form 2 June 2009

Veldhuis JD, Hudson SA, Bailey JN, Erickson D. Regulation of basal, pulsatile, and entropic (patterned) modes of GH secretion in a putatively low-somatostatin milieu in women. Am J Physiol Endocrinol Metab 297: E483–E489, 2009. First published June 2, 2009; doi:10.1152/ajpendo.00136.2009.—Somatostatin (SS) released by hypothalamic neurons inhibits GH exocytosis noncompetitively. Therefore, we postulated that attenuation of GH feedback-induced SS outflow would help to unmask covariates of endogenous secretagogue drive. To this end, 42 healthy pre- and postmenopausal women were randomly assigned to receive leuprolide plus estradiol (E2) or leuprolide plus placebo. A putatively low-SS milieu was imposed by 1-arginine infusion. Deconvolution and regularity analyses were applied to 6-h GH concentration-time profiles. By two-way ANOVA, age negatively (P < 0.001) and E2 positively (P = 0.001) determined pulsatile GH secretion in the presumptively SS-deficient milieu (P < 0.001). Comparable effects were exerted on the mass of GH secreted per burst per unit distribution volume (age: P = 0.001, E2: P < 0.001, overall P < 0.001). E2 alone predicted basal (nonpulsatile) GH secretion (P = 0.004). Stepwise forward-selection multivariate regression demonstrated that age (P = 0.0017) and E2 (P = 0.0002) together explained 46% of intersubject variability in pulsatile GH secretion (P < 0.001) and fully replaced the negative univariate effect of abdominal visceral fat (r² = 0.32, P < 0.001). Moreover, age and E2 (but not AVF) interacted to supervise GH regularity (P = 0.007). We conclude that age and E2 availability individually and together constitute primary predictors of basal, pulsatile, and patterned GH secretion in an inferentially feedback-silenced context in healthy women. Therefore, both factors must be considered in framing hypotheses of endogenous GH drive.

INTRAVENOUS INJECTION of a pulse of growth hormone (GH) exerts reversible negative feedback by evoking hypothalamic somatostatin (SS) outflow (1, 7, 37). SS acts by noncompetitively blocking exocytosis of GH secretory vesicles from somatotrope cells (29, 40). Hypothalamic SS release varies markedly on a short time scale in the rat, sheep, and pig (27, 35, 38, 39, 43). Fluctuations in SS outflow seem necessary for the generation of large GH pulses (21, 24, 33) but at the same time may confound acute GH responses to exogenous secretagogues such as GH-releasing hormone (GHRH) and ghrelin (a GH-releasing peptide) (8, 32, 34).

One direct SS receptor antagonist was tested in the rat (4), but none is available for use in humans. However, infusion of 1-arginine abolishes or significantly attenuates GH-induced as well as IGF-I-induced feedback inhibition of GHRH stimulation in humans (3, 15, 16). The capability of L-arginine to relieve negative feedback is prima facie evidence of a concomitant reduction in endogenous SS release or action (17, 48). Indeed, GH self-feedforward is also muted by guanidine in SS receptor subtype 2 knockout animals (52), 2) after immunoneutralization of SS (22, 26, 3) when electrolytic lesions are placed to isolate the mediobasal hypothalamus from periventricular SSergic inflow (3, 15, 16, 48), and 4) when the GH receptor is mutated or genetically downregulated in the brain (48). Although the exact biochemical mediator of the silencing action of GHRH on GH feedback remains unknown, infusion of a maximally effective amount of this amino acid evokes GH secretion and potentiates stimulation by GHRH and ghrelin (42, 45). Inasmuch as GHRH and ghrelin constitute two major stimulatory regulators of GH secretion (17, 48), exposure to 1-arginine should provide an indirect means to assess factors that determine endogenous GHRH and ghrelin drive (secretion and action).

When GH autoregulation is intact, age, estrogen availability, and relative adiposity [defined by body mass index (BMI) or abdominal visceral fat (AVF)] determine pulsatile GH secretion in a complex manner (17, 48). For example, estradiol (E2) amplifies the submaximally stimulatory effects (potencies) of GHRH and ghrelin (42, 45). Inversely, increased age and BMI/AVF reduce GH responses to most secretagogues. The extent to which the effects of age, estrogen, and adiposity are mediated via altered outflow of endogenous secretagogues compared with SS remains unclear. To address this point, the present study utilizes a triple paradigm comprising 1) infusion of 1-arginine to presumptively restrict GH- and IGF-I feedback-induced SS outflow, 2) evaluation of young [premenopausal (PRE)] and older [postmenopausal (POST)] women to appraise age-related effects, and 3) administration of a gondotropin-releasing hormone receptor agonist to downregulate the pituitary-ovarian axis before back placebo or a late follicular phase of E2 is added transdermally to clamp the estrogen milieu. The postulate is that this protocol will unmask the relative impact of age, estrogen, and AVF in a low-SS-mediated feedback milieu.

METHODS

Subjects. Women provided voluntary written informed consent approved by the Mayo Human Subjects Institutional Review Board. Exclusion criteria comprised sex steroid exposure within 1 mo of study; symptoms or signs of ischemic or inflammatory arteriovenous disease; acute or chronic hepatic, renal, immunologic, pulmonary, malignant, or infectious illness; cholelithiasis; known or suspected breast neoplasm; hemoglobin <11.8 g/dl; concomitant psychiatric treatment; drug or alcohol abuse; exposure to neuroactive drugs such as antidepressants, anxiolytics, or anticonvulsants within six

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half-lives; >3 kg weight change in 6 wk; regular nightshift work; transmigrid travel (>3 time zones traversed within the preceding 5 days); and inability to provide informed consent. Inclusion criteria were community-living, mentally competent, healthy women aged 18–30 or 50–80 yr. PRE status was defined by cyclic menses with a history of normal puberty. Pregnancy was excluded by serum human chorionic gonadotropin measurement. POST status was defined by amenorrhea for ≥2 yr, FSH >45 IU/l, LH >20 IU/l, and E2 ≤35 pg/ml (multiply by 3.68 for pmol/l). Computerized axial tomography at L4–L5 was used to estimate abdominal visceral fat, as described earlier (9).

Clinical protocol. The study was a prospectively randomized, parallel-cohort comparison of the effects of age and E2 on GH secretion during putatively reduced GH feedback-induced SS secretion. To achieve estrogen depletion, all 42 subjects received depot leuprolide acetate (3.75 mg im) twice wk apart (10). The first injection was given in younger volunteers within 8 days of menses onset and 48 h of a negative blood pregnancy test and ≥3 wk following discontinuation of sex hormone-containing contraception. Older women received their first injection ≥3 wk after withdrawal of any sex hormone supplements. Placebo patch (n = 10 PRE, n = 12 POST) vs. graded transdermal E2 repletion (n = 10 PRE, n = 10 POST) was accomplished on an outpatient basis starting on the day of the second leuprolide injection (day 0). In subjects randomized to receive estrogen, the initial transdermal E2 dose was 0.05 mg/day for 4 days. The dose was increased to 0.10, 0.15, and 0.20 mg/day every 4 days (Estraderm; Novartis, Basel, Switzerland). The highest E2 dose (0.2 mg/day) was administered for 10 days (days 14–23 inclusive).

L-arginine infusion and repetitive blood sampling were performed on days 17–23, when serum E2 concentrations were expected to be in the late-follicular phase range (9, 10). At the end of the study, micronized progesterone (100 mg orally) was administered for 12 days to women with an intact uterus according to standards of good medical practice.

Infusion and sampling schedule. At 1800 the night before the study, volunteers received a standardized outpatient meal of 8 kcal/kg distributed as 20% protein, 50% carbohydrate, and 30% fat. Subjects then remained fasting overnight and until the end of sampling. At 0700 the next morning, two intravenous (iv) catheters were placed in (contralateral) forearm veins to allow simultaneous L-arginine infusion and blood sampling (1 ml) every 10 min for 6 h from 0800 to 1400. The infusion comprised iv saline (20 ml/h) from 0800 to 1000 followed by L-arginine (30 g) delivered from 1000 to 1030 at a criterion (2). Outcomes evaluated were basal and pulsatile GH secretion, Subjects, investigators, and infusion administrators were masked until closure of the study. Prestudy power analyses predicted >90% statistical power to detect a 30% effect of age or E2 on unpaired two-tailed Student’s t-test if 40 subjects completed the study. The effects of age stratum and estrogen status and their interaction were evaluated using a two-way (2 × 2 factor) least-squares general-linear ANOVA model (51). Analysis of covariance was not used, since the putative covariate (mean 2-h prestimulus baseline GH concentration in each subject) was not significant. Departure of the variance-covariance matrix from compound symmetry was adjusted for the use of the Huynh-Feldt statistic. Wilk’s lambda was applied to evaluate the significance of possible interactions between age and status. The null hypothesis was that neither age stratum nor E2 condition determines GH secretion. Post hoc contrasts were made using Tukey’s honestly significantly different (HSD) test (13). Significance was construed for experiment-wise P < 0.05. Data are presented as means ± SE (n).

Regression analysis. Linear regression analysis was employed to explore correlations between GH secretion and age, E2 levels, and AVF or BMI. Stepwise, forward-selection, multivariate, linear regression analysis was applied to identify the principal determinant(s) of pulsatile GH secretion from among age, E2 concentration, and AVF in

Model-free analysis. Unstimulated GH concentrations were averaged over the 2-h saline-infusion interval (0800–1000) in each subject.

Deconvolution analysis. GH concentration time series (all 6 h) were analyzed using a recently developed automated deconvolution method, which was mathematically verified by direct statistical proof and empirically validated using hypothalamo-pituitary sampling and simulated pulsatile time series (6, 20). The MatLab-based algorithm first detrends the data and normalizes concentrations to the unit interval (0, 1) (19). Second, the program creates multiple successive potential pulse-time sets each containing one fewer via a smoothing process (a nonlinear adaptation of the heat-diffusion equation). Third, a maximum-likelihood expectation estimation method computes all secretion and elimination parameters simultaneously conditionally on each of the candidate pulse-time sets. Deconvolution parameters comprised basal secretion (β0), secretory burst mass (β0, 1, 1.1), random effects on burst mass (σβ), procedural/measurement error (σε), and a three-parameter flexible γ-secretory burst waveform (β1, β2, β3). The fast GH half-life was represented as 3.5 min constituting 37% of the decay amplitude and the slow half-life as 20.8 min (12). Statistical model selection was performed to distinguish among fits of the multiple candidate pulse-time sets using the Akaike information criterion (2). Outcomes evaluated were basal and pulsatile GH secretion (concentration units/session), mass secreted per burst (concentration units), and waveform shape (mode or time delay to maximal secretion after objectively estimated burst onset, min).

Statistical methods. The design was a prospectively randomized, placebo-controlled, masked parallel-cohort assessment of the effects of age and E2 on pulsatile GH secretion unleashed by L-arginine infusion. Subjects, investigators, and infusion administrators were masked until closure of the study.

Table 1. Hormone measurements during leuprolide/E2 and leuprolide/Pl clamps

<table>
<thead>
<tr>
<th>Subject Groups</th>
<th>E2, pg/ml</th>
<th>IGF-I, µg/l</th>
<th>IGFBP-1, µg/l</th>
<th>IGFBP-3, mg/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRE - E2 (n = 10)</td>
<td>11 ± 1.3a</td>
<td>352 ± 29a</td>
<td>14 ± 2.3a</td>
<td>5.1 ± 0.3a</td>
</tr>
<tr>
<td>PRE + E2 (n = 10)</td>
<td>8.8 ± 1.1a</td>
<td>187 ± 24a</td>
<td>22 ± 2.6ab</td>
<td>4.1 ± 0.2bc</td>
</tr>
<tr>
<td>POST - E2 (n = 12)</td>
<td>147 ± 7.3a</td>
<td>418 ± 46a</td>
<td>28 ± 2.4ab</td>
<td>4.8 ± 0.3bc</td>
</tr>
<tr>
<td>POST + E2 (n = 10)</td>
<td>116 ± 10a</td>
<td>176 ± 24a</td>
<td>38 ± 6.6b</td>
<td>3.4 ± 0.2a</td>
</tr>
<tr>
<td>P values</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.15</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Data are means ± SE. E2, estradiol; Pl, placebo; IGFBP-1 and -3, IGF-binding protein-1 and -3, respectively; PRE, premenopausal; POST, postmenopausal. *Multiply by 3.68 for pmol/l. †ANOVA within each column. Different (unshared) superscripted letters denote significantly different means by Tukey’s honestly significantly different post hoc test for multiple comparisons.
the combined cohorts (n = 42 subjects). Computations were made using Systat Version 11 (Systat, Point Richmond, CA).

**Approximate entropy.** Approximate entropy (ApEn) is a scale- and model-independent univariate regularity statistic used to quantify the orderliness (subpattern consistency) of serial measurements. Mathematical models and feedback experiments establish that pattern or-derliness monitors feedback and/or feedforward interactions within an interlinked axis with high sensitivity and specificity (both >90%) (31, 49).

**RESULTS**

PRE women had (means ± SE) ages and BMIs of 23 ± 0.62 and 25 ± 1.1 yr and 26 ± 0.75 and 23 ± 1.0 kg/m², respectively, in the placebo and E2 limbs. Corresponding values in POST women were 26 ± 0.75 and 23 ± 1.0 kg/m², respectively.

Table 1 shows the comparability of E2 concentrations in PRE and POST women subjected to a given low- or high-E2 clamp. IGF-I concentrations were lower in POST than in PRE women whether compared in the setting of leuprolide plus E2 or leuprolide plus placebo. IGFBP-1 was higher in POST than in PRE volunteers with comparable E2 status. Additionally, POST subjects supplemented with E2 had higher IGFBP-1 concentrations than PRE volunteers given placebo. IGFBP-3 was maximal in PRE E2 individuals. Exploratory univariate regression analysis indicated that fasting (2-h mean unstimu-lated) GH concentrations before L-arginine infusion correlated positively with E2 levels ($r^2 = 0.14, P = 0.015$), and negatively with AVF ($r^2 = 0.10, P = 0.041$), but not with age ($P = 0.21$). By stepwise forward-selection regression, E2 concentrations alone explained the effects of E2 and AVF on fasting unstimulated GH concentrations ($r^2 = 0.14, P = 0.015$).

Fig. 1. Age and estradiol (E2) determine pulsatile growth hormone (GH) secretion in a putatively low-somatostatin milieu. Two-way analysis of variance in 42 women given leuprolide with placebo or E2 addback transdermally and then intravenous infusion of L-arginine. Volunteers were sampled every 10 min for 6 h, which included a 2-h baseline. Tukey’s honestly significantly different (HSD) test was applied to compare means post hoc. $P$ values are as indicated. The number of subjects in each of the 4 groups is stated within each bar. Data are means ± SE. PRE, premenopausal; POST, postmenopausal.

Fig. 3. Impact of E2 and PRE vs. POST status on basal (unstimulated nonpulsatile) GH secretion in 42 women studied under a leuprolide clamp. The format is that of Fig. 1.

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Fig. 2. E2 augments the mass of GH secreted per burst in PRE and POST women, with ~3-fold greater responses in PRE than in POST individuals. Data are otherwise presented as described in Fig. 1.

Fig. 4. Univariate linear regression of pulsatile GH secretion unleashed by L-arginine infusion on abdominal visceral fat (top) or body mass index (bottom). Data were obtained from 42 women. $P$ values reflect Pearson’s correlation coefficient with corresponding $r^2$. 

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Pulsatile GH secretion was quantified by deconvolution analysis. Based upon two-way ANOVA, both age ($P < 0.001$) and $E_2$ status ($P = 0.001$) determined pulsatile GH responses to l-arginine infusion (overall $P < 0.001$; Fig. 1). Post hoc comparisons via Tukey’s HSD test revealed that maximal pulsatile GH secretion occurred in PRE + $E_2$ ($P = 0.016$ vs. POST + $E_2$, $P = 0.027$ vs. PRE - $E_2$, and $P < 0.001$ vs. POST - $E_2$). The interaction between age and $E_2$ trended toward significance ($P = 0.067$).

The opposing effects of age and $E_2$ on pulsatile GH secretion during putatively muted GH negative feedback were explicable by modulation of the mass of GH secreted per burst. According to two-way ANOVA, age had a significantly negative ($P = 0.001$) and $E_2$ a significantly positive ($P < 0.001$) effect (overall $P < 0.001$). Post hoc multiple-comparison contrasts revealed that GH secretory burst mass in PRE + $E_2$ significantly exceeded that in all three other study cohorts ($0.001 \leq P \leq 0.036$; Fig. 2).

Basal (nonpulsatile) GH secretion was determined statistically by $E_2$ ($P = 0.004$) but not by age ($P = 0.80$) (overall ANOVA, $P = 0.037$; Fig. 3). Basal secretion in PRE + $E_2$ women was similar to that in POST + $E_2$ individuals, and both values exceeded those in the two $E_2$-deficient cohorts. Basal secretion represented 1.4, 4.3, 3.8, and 12% of pulsatile GH secretion in the PRE - $E_2$, PRE + $E_2$, POST - $E_2$, and POST + $E_2$ categories, respectively.

Univariate linear regression analysis disclosed that l-arginine-induced pulsatile GH secretion correlated negatively with computerized tomography-estimated AVF ($P < 0.0001$) and $BMI$ ($P = 0.0012$), which explained 32 and 23%, respectively, of interindividual response variability (Fig. 4). Age was a strongly positive univariate predictor of AVF ($P < 0.0001$, $r^2 = 0.32$). Stepwise forward-selection multivariate regression analysis ($n = 42$ subjects) was used to assess the concomitant contribution(s) of age and $E_2$ (if any) to the effect of AVF. Figure 5 is three-dimensional plot of the concerted influences of age (negatively; $P = 0.0017$) and $E_2$ concentrations (positively; $P = 0.0002$) on stimulated pulsatile GH secretion ($P < 0.0001$ overall). In the multivariate regression model, AVF vanished as a contributor, leaving age and $E_2$ together to explain 46% of interindividual response variability. When GH secretory burst mass was used as the dependent variable, the effects of age ($P = 0.029$) and $E_2$ ($P = 0.0004$) were also significant (overall $P = 0.0001$), together accounting for 37% of intersubject variance. The mode (time delay in minutes from GH secretory burst onset to maximal secretion) was not significantly related to age, $E_2$, AVF, or BMI.

$ApEn$ was employed to quantify GH secretory pattern regularity after time series were detrended by first differencing (36). ANOVA of GH $ApEn$ values revealed a significant interaction between age and $E_2$ ($P = 0.007$) but no independent effects of age ($P = 0.23$) or $E_2$ ($P = 0.13$) (overall $P = 0.016$; Fig. 6). The interaction consisted of higher GH $ApEn$ (greater irregularity = less orderliness) in POST + $E_2$ than in PRE + $E_2$ ($P = 0.032$) and lower GH $ApEn$ (less irregularity) in PRE + $E_2$ than in PRE - $E_2$ ($P = 0.022$).

**DISCUSSION**

The present investigation evaluated the hypothesis that age, $E_2$ availability, and body composition modulate endogenous feedforward drive of pulsatile GH secretion in 42 healthy women. Key outcomes in the presumptively low-SS (low GH...
feedback) milieu were that 1) age and E2 concentrations prominently (each \( P \leq 0.001 \)) control pulsatile GH output with a trend toward an interaction (\( P = 0.067 \)); 2) the negative effect of age and the positive effect of E2 are mediated by corresponding changes in the size (mass) of GH secretory bursts (\( \mu \text{g of GH released/unit distribution volume per burst} \)); 3) a smaller positive effect of E2 operates on basal GH secretion (\( P = 0.004 \)), which represented 1.4–12% of pulsatile GH secretion; 4) indexes of body composition explain significant (AVF 32%, BMI 23%) variability in pulsatile GH secretion; 5) by stepwise forward-selection multivariate analysis, the univariate effects of AVF and BMI are completely explained by age and E2, together accounting for 46% of intersubject variability in feedback-disinhibited, burst-like GH secretion (\( P < 0.0001 \)); and 6) according to ApEn analysis, E2 enhances GH secretory pattern orderliness in PRE but not POST women under presumptively low-SS feedback. Measured E2 concentrations supported subject compliance with E2/placebo patches. Thus, the ensemble outcomes indicate that, to the extent that l-arginine mutes GH autoregulation, age and E2 represent major determinants of endogenous feedforward drive and, therefore, of physiological GHRH and ghrelin action.

Under inferably reduced GH feedback imposed by l-arginine infusion, pulsatile GH secretion was 2.7-fold lower in POST than in PRE women despite statistically similar E2 concentrations. The mean E2 value (133 pg/ml = 489 pmol/l) is comparable with that observed in the late follicular phase of the normal menstrual cycle, when pulsatile GH secretion increases about 2.2-fold in healthy PRE women (11, 28, 50). An explanatory postulate would be that factors associated with POST status or aging diminish estrogen’s capability to amplify endogenous GH secretory pattern orderliness and ghrelin’s drive of burst-like GH release. In contrast, compared with placebo, E2 elevated basal (nonpulsatile) GH secretion equally in PRE and POST women. These data suggest that distinct E2- and age-related mechanisms regulate burst-like and basal GH secretion. GH deficiency may contribute to bone loss and muscle wasting in advancing age (48). Whether increased GH production secondarily to E2 supplementation confers protection against catabolism has not been established. The question arises because E2 can also antagonize GH action on certain target tissues, such as liver (23), resulting in lower IGF-I and IGFBP-3 and higher IGFBP-1 concentration (44).

Univariate regression analysis identified a prominently negative correlation between AVF (or BMI) and low-feedback-associated pulsatile GH secretion. Notably, the independent covariates age and E2 together completely explained the statistical effect of AVF, possibly reflecting in part the positive correlation between age and AVF. However, whether age and AVF affect GH secretion via shared pathways has not yet been established.

ApEn is a regularity statistic that provides a barometer of the degree of network-like coordination driving orderly hormone output (14, 18). Earlier studies indicate that ApEn of GH release increases in midpuberty and after E2, testosterone, or GHRH administration (46). Increased ApEn signifies attenuation of feedback-coordinating signals on theoretical and empirical grounds (49). Under putatively low-GH feedback imposed by l-arginine infusion, ApEn of GH release was also high. Supplementation with E2 in PRE but not POST individuals reduced ApEn significantly, defining a more orderly GH release process (\( P = 0.007 \)). The basis for the impaired feedback-modifying effect of E2 in POST individuals is not known. However, estrogen’s enhancement of GH secretory regularity in PRE women is selective to the GH feedback-restricted milieu (30). This raises the possibility that E2 exposure evokes more regular GH secretion in PRE women by increasing feedforward by endogenous secretagogues.

Caveats include the somewhat small cohort size (\( n = 42 \)), the use of a single E2 addback concentration, the relatively short duration of estrogen deprivation and repletion, the possibility that l-arginine might exert unknown effects, and the need to extend these findings to men and children and to corroborate outcomes prospectively so as to define a causal effect of aging.

In conclusion, analyses of basal, pulsatile, and entropic modes of GH secretion in PRE and POST women studied under a low- or high-E2 clamp in a putatively low-GH feedback milieu disclose 1) prominent joint effects of age (negative) and E2 (positive) on the summed mass of GH secreted in bursts, 2) significant univariate effects of AVF and BMI (both negative) on the same measurement, 3) a positive impact of E2 on basal (nonpulsatile) GH secretion, and 4) an age-by-E2 interaction in controlling the regularity of the GH secretory process. The collective outcomes motivate consideration of more complex models of hypothalampituitary mechanisms regulating basal, pulsatile, and entropic GH secretion.

ACKNOWLEDGMENTS

We thank Donna Scott for capable support of manuscript preparation, Ashley Bryant for excellent data analysis and graphics, the Mayo Immunochanical Laboratory for assay assistance, and the Mayo research nursing staff for implementing the protocol.

GRANTS

This work was supported in part via Clinical Translational Research Center Grant MO1-RR-00555 to the Mayo Clinic and Foundation from the National Center for Research Resources (Rockville, MD) and R01-NIA-AG-29362, R21-DK-072095, and DK-063609 from the National Institutes of Health (Bethesda, MD).

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