Evidence that endogenous SST inhibits ACTH and ghrelin expression by independent pathways

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Corticosterone and total ghrelin levels are increased in somatostatin (SST) knockout mice (Sst−/−) compared with SST-intact controls (Sst+/+). Because exogenous ghrelin can increase glucocorticoids, the question arises whether elevated levels of ghrelin contribute to elevated corticosterone levels in Sst−/− mice. We report that Sst−/− mice had elevated mRNA levels for pituitary proopiomelanocortin (POMC), the precursor of adrenocorticotrophic hormone (ACTH), whereas mRNA levels for hypothalamic corticotropin-releasing hormone (CRH) did not differ from Sst+/+ mice. Furthermore, SST suppressed pituitary POMC mRNA levels and ACTH release in vitro independently of CRH actions. In contrast, it has been reported that ghrelin increases glucocorticoids via a central effect on CRH secretion and that n-octanoyl ghrelin is the form of ghrelin that activates the GHS-R1a and modulates CRH neuronal activity. Consistent with elevations in total ghrelin levels, Sst−/− mice displayed an increase in stomach ghrelin mRNA levels, whereas hypothalamic and pituitary expression of ghrelin was not altered. Despite the increase in total ghrelin levels, circulating levels of n-octanoyl ghrelin were not altered in Sst−/− mice. Because glucocorticoids and ghrelin increase in response to fasting, we examined the impact of fasting on the adrenal axis and ghrelin in Sst+/+ and Sst−/− mice and found that endogenous SST does not significantly contribute to this adaptive response. We conclude that endogenous SST inhibits basal ghrelin gene expression in a tissue specific manner and independently and directly inhibits pituitary ACTH synthesis and release. Thus endogenous SST exerts an inhibitory effect on ghrelin synthesis and on the adrenal axis through independent pathways.

somatostatin; adrenocorticotrophic hormone; hypothalamic-pituitary-adrenal axis; corticotropin-releasing hormone; pituitary; stomach

SOMATOSTATIN (SST), OR ITS SYNTHETIC ANALOGS, can reduce circulating adrenocorticotrophic hormone (ACTH) and glucocorticoid levels in rats and humans in vivo (19, 43, 46). The inhibitory actions of SST are believed to be due, at least in part, to a direct effect on the pituitary. This hypothesis is supported by the fact that SST can block corticotropin-releasing hormone (CRH)-mediated ACTH release in cultures of primary rat pituitary cells, mouse pituitary tumor cells (AtT-20), and cultures of human corticotropinomas (20, 25, 44), where this effect appears to be mediated by the SST receptors sst2 and/or sst5 (20, 43, 44). Both the in vivo and in vitro inhibitory effects of SST on ACTH release can be masked by glucocorticoids, where glucocorticoids directly inhibit basal and CRH-mediated ACTH release (19, 25). However, a recent study (20) demonstrates that an sst5 selective agonist can inhibit ACTH release in vitro in the presence of glucocorticoids. The inhibitory action of exogenous SST on ACTH release has prompted speculation regarding the role of endogenous SST as a corticotropin release-inhibiting factor (14). More recent observations support such a role, where pituitaries of sst2 knockout mice release more ACTH in vitro (47) and corticosterone levels are elevated in SST knockout mice (Sst−/−) (50).

Preliminary data generated by our laboratory have confirmed that basal corticosterone levels are elevated in both male and female Sst−/− mice (28). Interestingly, using an assay that recognizes both the n-octanoyl and des-acyl forms of ghrelin, we also found that total ghrelin levels of Sst−/− mice were 2-fold that of Sst+/+ controls. The n-octanoyl modified form of ghrelin, via activation of the GHS-R1a, has been shown to enhance food intake and stimulate the release of growth hormone and glucocorticoids, whereas the des-acyl form of ghrelin, via an unknown receptor pathway, has been shown to decrease food intake, regulate cardiac and adipocyte function, and mediate cell proliferation and apoptosis in a variety of tissues (for review see Refs. 9, 11, 16, 23, and 24). Because n-octanoyl ghrelin and its synthetic analogs [growth hormone secretagogues (GHS)] have been shown to increase ACTH and glucocorticoids in chickens, rats, mice, and humans (4, 5, 37, 45), where this action is thought to be mediated by an increase in CRH neuronal activity and CRH gene expression (5, 17, 21), we hypothesized that the elevated ghrelin levels may in part be responsible for the increase in corticosterone levels in the Sst−/− mice. However, it should be noted that the role of ghrelin in mediating the hypothalamic-pituitary-adrenal (HPA) axis may represent a pharmacological response given that a recent report (27) demonstrated that administration of ghrelin in fed subjects, to achieve circulating concentration observed just prior to anticipated food intake, did not affect circulating ACTH or cortisol levels.

Therefore, to further explore the interrelationship between endogenous SST, ghrelin, and adrenal function we compared the HPA axis of fed Sst+/+ and Sst−/− mice in relation to circulating ghrelin levels (measured as total or n-octanoyl only) and ghrelin gene expression in the stomach, pituitary, and hypothalamus. Because a rise in glucocorticoids and ghrelin represent a part of the adaptive response to fasting, we also examined the role endogenous SST plays in mediating this

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http://www.ajpendo.org
response. Finally, we examined the direct effect of SST and ghrelin on proopiomelanocortin (POMC) mRNA levels and ACTH release in primary pituitary cell cultures.

MATERIALS AND METHODS

Animals. Development and initial characterization of SST knockout (Sst−/−) mice have been previously reported (50). Male mice, heterozygous for the Sst-null mutation, were bred in a C57BL/6J background (Jackson Laboratory, Bar Harbor, ME) to generate Sst+/− and Sst−/− mice for this study. Genotypes were determined by PCR of tail-snip DNA, using primers and genotyping protocol reported in the Jackson Laboratory web site for the 129S-Ssttm1Ute/J background of tail-snip DNA, using primers and genotyping protocol reported in the Jackson Laboratory web site for the 129S-Ssttm1Ute/J background (http://jaxmice.jax.org/pub-cgi/protocols/protocols.sh?objtype=protocol &protocol_id=210). All experimental procedures were approved by the Animal Care and Use Committees of the University of Illinois at Chicago and the Jesse Brown Veteran’s Administration Medical Center. Animals were housed under a 12:12-h light-dark cycle (lights-on 0700). All mice were handled daily at least 1 wk prior to euthanasia to acclimate them to personnel and handling procedures.

In vivo studies. For studies examining the impact of endogenous SST on the adrenal axis and ghrelin, male mice 9–10 wk of age were weighed and food was withdrawn (0800–0900) from a subset of mice, whereas the remaining received food ad libitum (n = 4–10 mice/genotype/treatment group). Forty-eight hours later mice were weighed and killed by decapitation, without anesthesia. Trunk blood was mixed with 15 μl of MiniProtease inhibitor (Roche, Nutley, NJ) and placed on ice until centrifugation at 13,000 rpm for 10 min. Plasma was stored at −80°C until evaluation of circulating hormones. Hypothalami, pituitaries, and stomachs were collected and frozen in liquid nitrogen and stored at −80°C until analysis of mRNA levels by quantitative real-time RT-PCR (qRT-PCR, see below).

Primary pituitary cell cultures. For studies examining the direct effects of SST and ghrelin on pituitary POMC mRNA levels and ACTH release, pituitaries were collected from 10- to 12-wk-old male mice from a mixed background (C57BL/6J × FVB/N; n = 3–5 pituitaries pooled/experiment, 3 separate experiments) or randomly cycling female baboons ranging in age from 7 to 12 yr (n = 1 pituitary/experiment, 2–5 separate experiments). Mouse pituitaries were obtained after CO2 asphyxiation, whereas baboon pituitaries were obtained after pentobarbital sodium overdose from control animals under Institutional Animal Care and Use Committee-approved studies conducted by other University of Illinois at Chicago investigators. Pituitaries were enzymatically dispersed into single cells as previously described (2), with the exception that mouse pituitaries were dispersed in 1.7-ml polypropylene microfuge tubes (2–3 pituitaries/1 ml digestion medium/tube), and baboon pituitaries were dispersed in spinner flasks (1 pituitary/30 ml digestion medium/flask). All culture reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise specified. Cells were plated at 2 × 10⁷/well in α-MEM (Invitrogen, Grand Island, NY) containing 10% horse serum, 0.15% BSA, 6 mM HEPES, and penicillin-streptomycin (Invitrogen). After a 24-h incubation cultures were rinsed in serum free medium, and vehicle or SST (100 nM) and CRH (10 nM), alone or in combination, were added or cultures were treated with ghrelin (10 nM, 3–5 wells/treatment group). Cultures were incubated for an additional 18 h, and medium was removed and frozen for subsequent analysis of ACTH levels and total cellular RNA recovered for determination of POMC mRNA levels, as described below.

RNA isolation and reverse transcription. Total RNA was extracted from whole hypothalami and pituitaries or from primary pituitary cell cultures using the Absolutely RNA RT-PCR Miniprep kit (Stratagene, La Jolla, CA) with DNase treatment according to the manufacturer’s instructions. For RNA extraction from whole stomachs, the protocol was modified. Specifically, the stomach was placed in a 15-ml polypropylene conical tube containing 2 ml of lysis buffer. The sample was mechanically homogenized using a conical teflon pestle for ~5 min. Homogenates (1.5 ml) were transferred to 1.7-ml microfuge tubes and centrifuged at 13,000 rpm for 15 min, and 200 μl of the supernatant were mixed with 400 μl of lysis buffer. The solution was subjected to further homogenization for 1 min and centrifuged for 5 min, and supernatants were then column purified using the Absolutely RNA RT-PCR Miniprep kit as instructed. The amount of recovered RNA from hypothalami, pituitaries, and stomachs, was determined by the Ribogreen RNA quantification kit (Molecular Probes, Eugene, OR). Total RNA (1 μg for whole tissue extracts and 0.25 μg for primary pituitary cell cultures) was reversed transcribed (RT) in a 20-μl volume using random hexamer primers and reagents supplied in the cDNA First Strand Synthesis kit (MRI Fermentas, Hanover, MD). RT reactions were treated with Ribonuclease H (1 U, MRI Fermentas), and duplicate aliquots (1 μl) of the resulting CDNA were amplified by qRT-PCR, where samples are run against synthetic standards to estimate mRNA copy number (see below).

Primer selection. Specific primers for all mouse transcripts were designed using published sequences as templates [GenBank, National Center for Biotechnology Information (NCBI); Table 1]. To obtain partial nucleotide sequences of the baboon POMC and cyclophilin A coding region, we aligned published cDNA sequences from a variety

### Table 1. Species-specific primers used for quantitative real-time RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank Accession No.</th>
<th>Primer Sequence</th>
<th>Nucleotide Position</th>
<th>Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRH</td>
<td>NM_205769</td>
<td>Sense: TCTGGATCTCAGCCTCCACCT</td>
<td>Sn 630</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antisense: CACATCAGTTTCCTGGTTGCT</td>
<td>As 724</td>
<td></td>
</tr>
<tr>
<td>POMC</td>
<td>NM_008895</td>
<td>Sense: GAGGGCCTTCCCCCTAAGCTT</td>
<td>Sn 617</td>
<td>154</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antisense: CACCGTAACGCTGGTCTTCCCT</td>
<td>As 770</td>
<td></td>
</tr>
<tr>
<td>Ghrelin</td>
<td>NM_021488</td>
<td>Sense: TCCAGAAGGCCACGACTAA</td>
<td>Sn 164</td>
<td>126</td>
</tr>
<tr>
<td>SST</td>
<td>NM_009215</td>
<td>Sense: AACATCGAAGGAGCATGGA</td>
<td>Sn 289</td>
<td>113</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antisense: TGGCTCTTGGAGAAGGAAAG</td>
<td>Sn 421</td>
<td>109</td>
</tr>
<tr>
<td>Cyclophilin A</td>
<td>NM_008907</td>
<td>Sense: TGTCAGACGTCGAAATGCT</td>
<td>Sn 529</td>
<td></td>
</tr>
<tr>
<td>Baboon</td>
<td>POMC</td>
<td>Sense: CCCTACAGGATGGAGACCTT</td>
<td>Sn 7</td>
<td>127</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antisense: CTGCTGTGTGTGTGTGT</td>
<td>As 133</td>
<td></td>
</tr>
<tr>
<td>Cyclophilin A</td>
<td>DQ315472</td>
<td>Sense: CAAGAAGGATGGGTGAGTTT</td>
<td>Sn 351</td>
<td>122</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antisense: TGTCAGACGTCGAAATGCT</td>
<td>As 472</td>
<td></td>
</tr>
</tbody>
</table>

CRH, corticotropin-releasing hormone; POMC, proopiomelanocortin; SST, somatostatin.
of several primate species [human, Pan troglodytes (chimpanzee) and Macaca nemestrina (pig-tailed macaque)] and selected primers corresponding to areas of 100% homology. Using these primers, we amplified cDNA generated from baboon pituitary total RNA. The product generated was sequenced and demonstrated close similarity to POMC and cyclophilin A transcripts of other primates. This sequence was submitted to GenBank, and the accession numbers, which are shown in Table 1, were subsequently used to select primers appropriate for real-time PCR. Specifically, primers were selected using Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi; Steve Rozen, Whitehead Institute for Biomedical Research) with selection parameters set to 1) pick primers that span an intron (when known) and that differ by no more than 0.2°C in annealing temperature, 2) exclude primers that may form primer dimers, and 3) amplify a product of 90–200 bp. Sequences of selected primers were used in BLAST (NCBI) searches to check for potential homology to sequences other than the designated target. Primers were then used in a standard PCR reaction to amplify cDNA generated by reverse transcription, and products were run on agarose gels and stained with ethidium bromide to confirm that only one band, of the expected size, was amplified and no primer dimers formed. These PCR products were then column purified (Qiagen, Valencia, CA) and sequenced to confirm target specificity. The primers, the expected product sizes, annealing temperatures and Genbank accession numbers are provided in Table 1.

Confirmation of primer efficiency, construction of standard curves, and qRT-PCR. The initial screening of primer efficiency in a real-time PCR reaction was performed amplifying twofold dilutions of RT products, where optimal efficiency was demonstrated by a difference of one cycle threshold between dilutions. At the end of the amplification, the final products were subjected to graded temperature-dependent dissociation to verify that only one product was amplified. For real-time PCR reaction, IQ SYBR Green Supermix (Bio-Rad, Hercules, CA) was used, where thermostating and fluorescence detection were performed using a Stratagene Mx3000p real-time PCR machine. If preliminary primer efficiency tests were confirmed, the concentration of purified PCR products (generated by PCR) was amplified and no primer dimers formed. These PCR products were then column purified (Qiagen, Valencia, CA) and sequenced to confirm target specificity. The primers, the expected product sizes, annealing temperatures and Genbank accession numbers are provided in Table 1.

Table 2. Absolute cDNA copy number/0.05 μg total RNA of gene transcripts in the hypothalamus, pituitary, and stomach of fed and fasted (48 h) Sst+/− and Sst−/− mice, as determined by quantitative real-time RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sst+/− Fed</th>
<th>Sst+/− Fasted</th>
<th>Sst−/− Fed</th>
<th>Sst−/− Fasted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypothalamus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclophilin A</td>
<td>204,279 ± 36,352</td>
<td>235,119 ± 35,305</td>
<td>206,973 ± 22,778</td>
<td>252,667 ± 44,545</td>
</tr>
<tr>
<td>SST</td>
<td>37,878 ± 3,614</td>
<td>36,490 ± 7,383</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CRH</td>
<td>80 ± 11</td>
<td>124 ± 27</td>
<td>67 ± 9</td>
<td>116 ± 18</td>
</tr>
<tr>
<td>Ghrelin</td>
<td>17 ± 2</td>
<td>18 ± 2</td>
<td>17 ± 3</td>
<td>23 ± 6</td>
</tr>
<tr>
<td>Pituitary</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclophilin A</td>
<td>130,493 ± 18,570</td>
<td>123,535 ± 9,931</td>
<td>131,479 ± 15,341</td>
<td>125,613 ± 7,300</td>
</tr>
<tr>
<td>POMC</td>
<td>1,875,000 ± 275,984</td>
<td>1,414,520 ± 97,762</td>
<td>2,810,857 ± 123,729</td>
<td>2,110,800 ± 172,841</td>
</tr>
<tr>
<td>Ghrelin</td>
<td>30 ± 4</td>
<td>67 ± 9</td>
<td>40 ± 6</td>
<td>70 ± 7</td>
</tr>
<tr>
<td>Stomach</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclophilin A</td>
<td>157,686 ± 18,046</td>
<td>178,200 ± 9,342</td>
<td>155,513 ± 10,126</td>
<td>149,350 ± 6,674</td>
</tr>
<tr>
<td>SST</td>
<td>55,687 ± 6,754</td>
<td>45,597 ± 10,466</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Ghrelin</td>
<td>170,228 ± 37,406</td>
<td>249,320 ± 34,145</td>
<td>336,713 ± 37,421</td>
<td>304,650 ± 28,158</td>
</tr>
</tbody>
</table>

Values represent means ± SE. ND, not detectable.
SST mRNA levels were undetectable by qRT-PCR in Sst/H11002/H11002 mice in both the hypothalamus (Fig. 1A) and stomach (Fig. 1B), thus confirming the PCR genotyping results. In Sst/H11001/H11001 mice, fasting tended to suppress hypothalamic and stomach SST mRNA levels; however, these effects did not reach statistical significance. A modest inhibitory effect of fasting on hypothalamic SST expression has been previously reported in mice (35). However, in the rat, fasting has been reported to enhance SST mRNA levels in the pyloric antrum but not the acid-secreting region of the stomach (38, 48), although others report a regional increase or decrease in SST mRNA following fasting, depending on the housekeeping gene used as the internal control (49).

**HPA axis of Sst/+/+ vs. Sst−/− mice.** Circulating corticosterone levels were significantly elevated in Sst−/− mice compared with wild-type controls (Fig. 2A). Forty-eight hours of fasting increased corticosterone levels, where this rise did not significantly differ between genotype. Elevated corticosterone levels of fed Sst−/− mice were associated with a significant increase in pituitary POMC mRNA levels compared with Sst+/+ mice (Fig. 2B). It should be noted that the differences in pituitary POMC expression were maintained between genotypes following the 48-h fast. Despite the elevated levels of POMC mRNA, Sst−/− mice tended to have lower levels of hypothalamic CRH mRNA compared with Sst+/+ mice, consistent with corticosterone negative feedback; however, this did not reach statistical significance (P < 0.17). However, fasting did result in an overall increase in CRH mRNA levels, independent of genotype (P < 0.05; Fig. 2C).

**Effect of SST on POMC mRNA levels and ACTH release in primary pituitary cell cultures.** The enhanced levels of pituitary POMC mRNA of Sst−/− mice occurred independently of increases in hypothalamic CRH expression, suggesting SST may directly mediate pituitary ACTH synthesis. To further explore this possibility, we examined the effects of SST (100 nM, 18 h) on POMC mRNA levels and ACTH release in primary mouse pituitary cell cultures in the presence and absence of CRH (10 nM), and the results are presented in Fig. 3A. In the mouse, SST alone decreased POMC mRNA levels.
and ACTH release. Although it has been previously reported that CRH enhances POMC mRNA levels in primary pituitary cell cultures (12, 15), CRH did not alter POMC expression in mouse pituitary cultures but did stimulate ACTH release, where this action was blunted by coculture with SST. A similar dissociation between the direct pituitary effects of CRH on POMC mRNA levels and ACTH release has been previously reported in the sheep (26). However, it should also be noted that CRH has been shown to enhance POMC mRNA levels in AtT20 cells, an ACTH-producing tumor cell line derived from a mouse pituitary (1). Therefore, the effects of CRH on POMC expression not only vary between species but are also dependent on the pathophysiological state of the tissue tested.

To determine whether the inhibitory action of SST on POMC expression and ACTH release is confined to the mouse model, or represents a regulatory control mechanism that can be observed across species, we repeated the in vitro experiment using primary pituitary cell cultures from a nonhuman primate (Papio anubis, baboon), and the results are shown in Fig. 3. The elevation in circulating total ghrelin levels was dramatically elevated in Sst−/− mice in both the fed and fasted state (Fig. 4A). The elevation in circulating total ghrelin levels of Sst−/− mice was associated with a significant increase in ghrelin mRNA levels in the stomach (Fig. 4C). Although Sst−/− mice displayed a clear increase in ghrelin expression, circulating levels of n-octanoyl ghrelin did not differ between genotype (Fig. 4B). Interestingly, fasting had an overall stimulatory effect on n-octanoyl ghrelin levels, where the response reached significance in the Sst+/+ mice (Fig. 4B). However, total ghrelin levels (Fig. 4A) and stomach ghrelin mRNA levels (Fig. 4C) were not significantly altered by fasting.

Although the stomach is the primary source of circulating ghrelin (3), ghrelin has been detected in both the hypothalamus (39) and pituitary (22). Therefore, we also examined whether endogenous SST and fasting interact to regulate hypothalamic and pituitary expression of ghrelin. The hypothalamus and pituitary did express detectable levels of ghrelin (Table 2). However, when hypothalamic and pituitary ghrelin mRNA levels were compared with those expressed in the stomach, the absolute mRNA copy number was 10,000- and 5,000-fold less, respectively. Hypothalamic ghrelin mRNA levels did not differ between genotype or in response to fasting (Fig. 5A), inconsistent with reports in the rat, where hypothalamic ghrelin mRNA levels were reduced by 24- and 48-h fasting (39). At the level of the pituitary, ghrelin mRNA levels did not differ between genotype; however, fasting significantly increased pituitary ghrelin expression in both groups (Fig. 5B).

Effect of ghrelin on POMC mRNA levels and ACTH release in primary pituitary cell cultures. It has been previously shown that ghrelin (n-octanoyl) or its synthetic analogs do not affect...
the release of ACTH from primary rat pituitary cell cultures or primary cultures from human fetal pituitaries (13, 41). However, a synthetic ghrelin analog was effective in stimulating ACTH release in cultures from human corticotropomas (7). Given that the direct pituitary effects of ghrelin on ACTH release may be dependent on the species, age, and physiological state of the tissue donor, we tested the direct effects of ghrelin (10 nM) on POMC mRNA levels and ACTH release in primary pituitary cell cultures from normal mice and baboons, and the results are shown in Fig. 6. Ghrelin had no significant effect on POMC mRNA levels in pituitary cell cultures prepared from mice or baboons. Likewise, ghrelin did not affect ACTH release from mouse pituitary cells. However, ghrelin did have a modest but significant stimulatory effect on ACTH release in baboon pituitary cell cultures, where the values shown are the means of five separate experiments, performed on cultures from different baboons. In three experiments ghrelin had no effect on ACTH release, whereas in two experiments ghrelin showed a clear 60% increase in ACTH released into the medium over the 18-h culture period (data not shown). Given that these pituitary cell cultures were prepared from randomly cycling female baboons of different ages, we cannot exclude the possibility that reproductive environment may alter the corticotrope response to ghrelin.

DISCUSSION

In Sst⁻/⁻ mice, corticosterone levels were more than doubled compared with their respective Sst⁺/+ control values, as previously reported (50). In this study, we demonstrate that elevation in glucocorticoid levels in the Sst⁻/⁻ mouse are associated with a significant increase in pituitary POMC expression, without a concomitant rise in hypothalamic CRH expression. These in vivo results, coupled with our present in vitro findings showing that SST can directly suppress POMC mRNA levels and ACTH release in primary mouse pituitary cell cultures and with the fact that pituitaries of sst2 knockout mice release more ACTH in vitro (47), strongly support the hypothesis that, at least in the mouse, endogenous SST can act as a corticotropin synthesis/release-inhibiting factor (14). The...
fact that the direct inhibitory actions of SST on POMC mRNA levels and ACTH release were also observed in primary pituitary cell cultures from a nonhuman primate suggests that endogenous SST may also play an important role in regulating corticotroph function in higher-order mammalian species, such as humans.

We also observed that total circulating ghrelin and stomach ghrelin mRNA levels [the primary source of circulating ghrelin (3)] were increased in Sst\(^{-/-}\) mice, indicating that endogenous SST tone is a critical regulator of ghrelin synthesis. This is consistent with reports demonstrating that treatment with SST or its synthetic analog (octreotide) suppresses circulating ghrelin in rats and humans (6, 34, 42), where this effect is thought to be primarily mediated via sst2 (42). The inhibitory effect of SST on ghrelin release can also be observed following perfusion of SST into the gastric artery, suggesting a direct effect on gastric ghrelin production (40). In fact, SST produced locally in the stomach may play a major role in mediating ghrelin synthesis and release, given that SST-producing gastric cells directly contact ghrelin-producing cells (40). In contrast to the role of endogenous SST in regulating gastric ghrelin expression, hypothalamic and pituitary ghrelin mRNA levels were not altered in the Sst\(^{-/-}\) mice compared with Sst\(^{+/+}\) controls, demonstrating that the regulatory action of SST on ghrelin expression is tissue specific.

Despite the rise in total ghrelin output, n-octanoyl ghrelin levels were unaltered in Sst\(^{-/-}\) mice. Also, fasting resulted in a rise in n-octanoyl ghrelin in both Sst\(^{+/+}\) and Sst\(^{-/-}\) mice without significant changes in total circulating ghrelin levels or stomach ghrelin gene expression, consistent with reports of others showing no effect of fasting on total circulating ghrelin levels in the mouse (29, 36). This is in contrast to the effects of fasting in rats, where increases in circulating levels of n-octanoyl ghrelin are clearly reflected by increases in total ghrelin levels, where \(\sim 90\%\) represents the des-acyl form (31).

Our present results demonstrate that regulation of ghrelin gene expression and posttranslational modification of ghrelin, via its acylation, is not always linked. This disconnection has also been observed in adult mice following feeding of C:8 medium-chain fatty acids, where n-octanoyl ghrelin levels rose, in the absence of changes in total ghrelin levels (32). Also, n-octanoyl ghrelin levels, but not total ghrelin levels, are reported to fall during the transition from suckling to weaning in mice and rats, where milk is rich in medium-chain fatty acids (33). Taken together, these results indicate that dietary medium-chain fatty acids can be used as direct substrates for acylation of existing ghrelin. Although the specific enzymatic pathway mediating acylation of ghrelin remains to be defined, we can conclude from our present results that endogenous SST tone has no major impact on this process in fed or fasted mice.

We originally hypothesized that the elevated ghrelin levels observed in the Sst\(^{-/-}\) mouse may contribute to the elevated glucocorticoids observed in this mutant model. Previous studies indicate that the stimulatory effect of ghrelin on the adrenal axis of the rat is not mediated by direct pituitary effects on ACTH secretion (13), consistent with our present in vitro results where primary mouse pituitary cell cultures were used. Also, ghrelin does not appear to have a direct effect on adrenal steroidogenesis (8). However, the major impact of ghrelin on glucocorticoid production is likely mediated centrally by enhancing CRH neuronal activity via neuropeptide Y-mediated suppression of GABA inhibitory tone (10). Given that the stimulatory effect of ghrelin on CRH release requires its acyl modification (30), we might conclude that SST-mediated alteration in total circulating ghrelin levels is not responsible for alterations in the adrenal axis of the Sst\(^{-/-}\) mice. We cannot ignore the possibility that centrally produced n-octanoyl ghrelin may be regulated by SST (39). If hypothalamic n-octanoyl

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**Fig. 6.** Effect of 18-h treatment of n-octanoyl ghrelin (10 nM) on POMC mRNA levels and ACTH release in primary pituitary cell cultures from mice (A) and baboons (B). POMC mRNA copy numbers were determined by qRT-PCR, and the values were adjusted by cyclophilin A copy number as an internal control. Values are expressed as % vehicle-treated controls (set at 100%) within experiment and represent means \(\pm SE\) of 3 independent experiments for mice (4–5 wells/treatment/experiment) and 5 independent experiments for baboons (4–6 replicates/treatment/experiment). In this set of experiments, the amount of ACTH released per \(2 \times 10^6\) cells over the 18-h period in vehicle-treated cultures was 1,792 \(\pm 68\) pg/ml for mouse and 4,476 \(\pm 1,050\) pg/ml for baboon. *Values that differ from vehicle-treated controls. \(P < 0.05\) was considered significant.
ghrelin levels were modulated by endogenous SST, we might have anticipated a change in CRH expression in the Sst−/− on the basis of previous observations (5, 21) showing that intracerebroventricular delivery of ghrelin or GH-releasing peptide-6 increases CRH mRNA levels in mice. However, the lack of endogenous SST had no stimulatory effect on hypothalamic CRH expression.

Taken together, these findings do not support our original hypothesis that elevated levels of ghrelin contribute to elevated corticosterone levels in the Sst−/− mouse. However, our findings do support a role for endogenous SST as a true corticotropin release-inhibiting factor under basal (fed) conditions, although endogenous SST does not play a major role in regulating the HPA axis in the fasted state. Our results also reveal that endogenous SST suppresses gastrointestinal ghrelin production; however, SST does not play a role in regulating posttranslational acylation of ghrelin in the fed or fasted state.

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REFERENCES

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