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Mechanisms Underlying the Diminished Sensitivity to Prolactin Negative Feedback during Lactation: Reduced STAT5 Signaling and Up-Regulation of Cytokine-Inducible SH2 Domain-Containing Protein (CIS) Expression in Tuberoinfundibular Dopaminergic Neurons

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Hyperprolactinaemia during lactation is a consequence of the sucking stimulus and in part due to reduced prolactin (PRL) negative feedback. To date, the mechanisms involved in this diminished sensitivity to PRL feedback are unknown but may involve changes in PRL signal transduction within tuberoinfundibular dopaminergic (TIDA) neurons. Therefore, we investigated signal transducers and activators of transcription (STAT) 5 signaling in the TIDA neurons of lactating rats. Dual-label confocal immunofluorescence studies were used to determine the intracellular distribution of STAT5 within TIDA neurons in the dorsomedial arcuate nucleus. In lactating rats with pups removed for 16 h, injection of ovine PRL significantly ($P < 0.05$) increased the STAT5 nuclear/cytoplasmic ratio compared with vehicle-treated mothers. In contrast, ovine PRL injection did not increase the STAT5 nuclear/cytoplasmic ratio in lactating mothers with pups, demonstrating that PRL signal transduction through STAT5 is reduced in TIDA neurons in the presence of pups. To investigate possible mechanisms involved in reduced PRL signaling, we examined the expression of suppressors of cytokine signaling (SOCS) proteins. Northern analysis on whole hypothalamus showed that CIS (cytokine-inducible SH2 domain-containing protein), but not SOCS1 or SOCS3, mRNA expression was significantly ($P < 0.01$) up-regulated in suckled lactating rats. Semiquantitative RT-PCR on arcuate nucleus micropunches also showed up-regulation of CIS transcripts. Immunofluorescence studies demonstrated that CIS is expressed in all TIDA neurons in the dorsomedial arcuate nucleus, and the intensity of CIS staining in these neurons is significantly ($P < 0.05$) increased in lactating rats with sucking pups. Together, these results support the hypothesis that loss of sensitivity to PRL-negative feedback during lactation is a result of increased CIS expression in TIDA neurons. (*Endocrinology* 147: 1195–1202, 2006)

PLASMA PROLACTIN (PRL) concentrations are controlled via a classical negative feedback loop (1). PRL is under predominantly inhibitory control by the catecholamine dopamine synthesized by the tuberoinfundibular dopaminergic (TIDA) neurons, most of which are localized to the dorsomedial arcuate nucleus of the hypothalamus. These neurons project to the median eminence where dopamine is released into hypophysial portal blood, and after reaching the anterior pituitary acts upon dopamine D2 receptors on lactotrophs to inhibit the synthesis and release of PRL. In turn, PRL acts in a short-loop negative feedback manner to regulate the synthesis and secretion of dopamine by the TIDA neurons. Many studies have shown that the activity of the TIDA neurons (as determined by the rate of dopamine synthesis, dopamine turnover, or dopamine concentrations in hypophysial portal blood) is increased when plasma PRL levels are increased by pharmacological manipulation (reviewed in Refs. 2 and 3). This negative short-loop feedback between PRL and dopamine is well recognized and is responsible for PRL homeostasis in most physiological states. However, the mechanisms by which PRL exerts negative feedback are relatively unknown, although PRL receptors are expressed on TIDA neurons (4–6), suggesting a direct effect.

One of the major intracellular signaling pathways for PRL receptors involves activation of Janus tyrosine kinase 2 (Jak2) and subsequent phosphorylation of signal transducers and activators of transcription (STAT) proteins (for review see Refs. 1 and 7). These phosphoSTAT proteins undergo movement to the nucleus (nuclear translocation) and bind to STAT response elements on promoter regions of genes to regulate transcription. Recent evidence supports this signaling pathway as part of PRL negative feedback in TIDA neurons. PRL is known to cause nuclear translocation of STAT5 in TIDA neurons in ovariectomized rats (8), and the presence of the

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Abbreviations: CIS, Cytokine-inducible SH2-domain-containing protein; FITC, fluorescein isothiocyanate; Jak2, Janus tyrosine kinase 2; N/C, nuclear/cytoplasmic; oPRL, ovine PRL; PRL, prolactin; SOCS, suppressors of cytokine signaling; RT, reverse transcription; STAT, signal transducers and activators of transcription; TIDA, tuberoinfundibular dopaminergic.

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STAT5b isoform appears to be critical for normal PRL negative feedback because STAT5b-deficient mice have abnormally high serum PRL concentrations (9).

In lactating females, chronically elevated plasma PRL concentrations are observed, a phenomena well known to be associated with the sucking stimulus. In the presence of the sucking pups, the TIDA neurons display relatively low activity (10, 11), and by mid-lactation the TIDA neurons are unresponsive to exogenous PRL (11, 12). In contrast, removal of pups and therefore the sucking stimulus results in increased TIDA neuronal activity, suppression of plasma PRL concentrations, and increased responsiveness of the TIDA neurons to exogenous PRL (12). The cellular mechanisms involved in these apparent changes in sensitivity to PRL negative feedback in mid-lactation remain unknown. No evidence exists to support the notion of down-regulation of PRL receptors in the arcuate nucleus during lactation; rather, they appear to be up-regulated (13). Nevertheless, resistance to PRL within the TIDA neurons could still occur through inhibition of intracellular signaling pathways. So, in the first part of our study, we examined PRL signaling through STAT5 in TIDA neurons of lactating rats. Results from these experiments indicated that PRL-induced STAT5 nuclear translocation in TIDA neurons is indeed reduced by the sucking stimulus. One possible explanation for this inhibition could be increased expression of a negative regulator of PRL signaling within the TIDA neurons. In this regard, certain members of the suppressors of cytokine signaling (SOCS) family of proteins, SOCS1, SOCS2, SOCS3, and cytokine-inducible SH2-domain-containing protein (CIS), are known to negatively regulate PRL receptor signaling through Jak2/STAT5 (14). Therefore, we next examined the hypothesis that loss of sensitivity to PRL negative feedback during lactation is caused by increased expression of a SOCS protein in TIDA neurons, which inhibits PRL signal transduction through STAT5.

Materials and Methods

Materials

Ovine PRL (oPRL-20, AFP-10677C) was obtained from Dr. A. F. Parlow and the National Hormone and Peptide Program (Torrance, CA). Bromocriptine mesylate (Parloldel) was obtained from Sandoz (North Ryde, Australia). Primary antibodies used for immunohistochemistry were rabbit anti-STAT5 (sc835X), rabbit anti-CIS (sc15344), and goat anti-SOCS3 (sc7009) from Santa Cruz Biotechnology (Santa Cruz, CA), and mouse antityrosine hydroxylase monoclonal (MAB-318) from Chemicon International (Temecula, CA). Secondary antibodies were donkey antirabbit Texas Red conjugate (711-075-152) and donkey antimouse fluorescein isothiocyanate (FITC) conjugate (715-095-151) from Jackson ImmunoResearch Laboratories (West Grove, PA), and biotinylated donkey antigoat IgG from Amersham Pharmacia Biotech (Buckinghamshire, UK). Streptavidin Texas Red conjugate (016-070-084z) was purchased from Jackson ImmunoResearch Laboratories. Trizol reagent for RNA extraction and Superscript Preamplification System for reverse transcription (RT) were obtained from Life Technologies, Inc. (Melbourne, Australia). PCR reagents including *Taq* polymerase were obtained from Biotech International (Perth, Australia).

Animals

Female Wistar rats obtained from the Central Animal Breeding House, University of Queensland were used. Rats were given free access to water and food and exposed to a 12-h light, 12-h dark photoperiod. Experiments were performed on cyclic (diestrus) and lactating rats (d 9–11 postpartum). Vaginal smears were taken each morning from cyclic rats to determine the stage of the estrous cycle, and animals were killed on the morning of diestrus after two normal estrous cycles. Late pregnant rats were inspected daily for births, and within 48 h of birth the number of pups was standardized to 12 pups/dam. Before death on d 9–11 postpartum, some of the lactating rats had their pups removed for 16 h, whereas the remainder continued to suckle.

In experiments where PRL was used, 1 h before death rats received a sc injection of 250 μg oPRL dissolved in 250 μl of oPRL buffer [30 mM $NaHCO_3$ in 150 mM NaCl (pH 10.8)] and 250 μl saline, or vehicle alone (250 μl oPRL buffer and 250 μl saline). In one experiment, bromocriptine mesylate (3 mg/kg in 1% tartaric acid/30% ethanol) was given by sc injection 2 h before oPRL or vehicle injection to acutely suppress endogenous PRL levels. This bromocriptine treatment is known to suppress the suckling induced increase in PRL in lactating rats (15), and acutely (within 2 h) reduce circulating serum PRL concentrations in ovariectomized rats (16).

All experiments were conducted in accord with NHMRC (Australia) guidelines and were approved by the Animal Experimentation and Ethics Committee of The University of Queensland.

Immunohistochemistry

Rats were killed by injection of pentobarbitone. The head was perfused with 100 ml of 1.0% sodium nitrite solution in 0.1 M sodium phosphate buffer (PB, pH 7.4), followed by 400 ml fixative (4% formaldehyde in 0.1 M PB, pH 7.4). The brain was removed and postfixed for 2 h, before being equilibrated in 30% sucrose in 0.1 M PB. After equilibration, the brain was embedded and frozen in OCT. Coronal sections (25 μm) of the hypothalamus were obtained using a cryostat. Sections were collected into tissue culture plates containing 0.1 M PB with 0.01% sodium azide and stored at 4 C until required.

Coronal sections located between bregma −2.30 and −3.30 (17) were examined. Free floating sections were washed in 0.1 M PBS (PBS; 3 × 5 min), then serum blocked for 20 min (10% horse serum in PBS containing 0.4% Triton X-100), before being incubated with primary antibodies; mouse antityrosine hydroxylase (1:1000), together with either rabbit anti-STAT5 (1:1000) or rabbit anti-CIS (1:20) or goat anti-SOCS3 (1:50) for 24 h at 4 C. All antibodies were diluted in buffer (2% horse serum, 0.4% Triton X-100 in 0.1 M PBS). After further washing in PBS (3 × 5 min), sections were incubated with secondary antibodies. For CIS and TH staining, donkey antirabbit Texas Red and donkey antimouse FITC were used together both at 1:400 dilution in antibody buffer for 18 h at 4 C. For SOCS3 and TH staining, biotinylated donkey antigoat IgG and donkey antimouse FITC were used (both at 1:200 dilution), followed by streptavidin Texas Red conjugate (1:200 dilution), with both incubations for 3 h at room temperature. Sections were again washed in PBS, before being mounted on glass microscope slides, and coverslipped under 50% glycerol in PBS. Control sections with omission of primary or secondary antibodies were performed to confirm specificity of the antisera. CIS and SOCS3 immunoreactivity was verified using other primary antibodies; rabbit anti-CIS (gift from A. Yoshimura, Fukuoka, Japan) and rabbit anti-SOCS3 (gift from D. J. Hilton, Melbourne, Australia).

Confocal immunofluorescence images were obtained as previously described (18). Diestrous rats, continuously suckled lactating rats, and lactating rats with pups removed 16 h before death were examined (n = 4 per group). During each confocal session, images were obtained from one animal in each treatment group in a blind unbiased manner. For quantitation, confocal settings were kept constant (aperture size, laser power, detector gain, zero black level adjustment) and captured images were analyzed using NIH image software (National Institutes of Health, Bethesda, MD).

The intracellular localization of STAT5 in TH-positive neurons in the dorsomedial arcuate nucleus (dmArc) was determined using the ratio of mean STAT5 fluorescence (Texas Red) intensity in the nucleus expressed as a proportion of that over the cytoplasmic profile of a TH (FITC) immunoreactive neuron [nuclear/cytoplasmic (N/C) ratio]. Where visible, the nucleolus was excluded from quantitation. For each animal, the STAT5 N/C ratio was determined from at least 30 neurons in the dmArc. Furthermore, the distribution of STAT5 immunostaining in dopaminergic neurons located in the zona incerta (present on the same coronal section as the dmArc) was also determined as a negative control because these neurons do not express the PRL receptor (19).

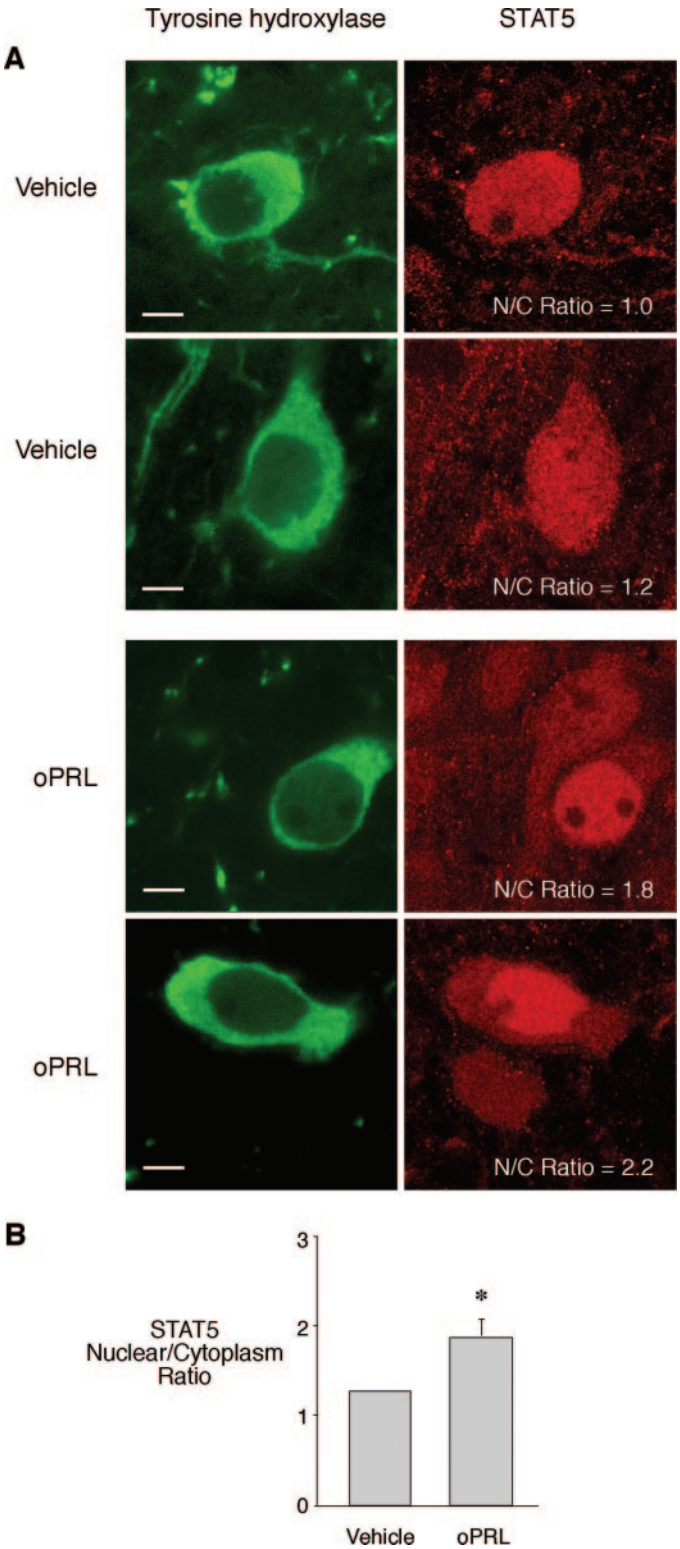

FIG. 1. Cellular localization of STAT5 in TIDA neurons of diestrous rats treated with sc injection of vehicle or oPRL (250 μg) given 1 h before death. A, Confocal images of four TIDA neurons in the dmArc, each from a different animal, showing the localization of tyrosine hydroxylase (*left panel*, FITC stain) and STAT5 (*right panel*, Texas Red stain). The STAT5 immunofluorescence in TIDA neurons of vehicle-treated animals is of similar intensity in the cytoplasmic and nuclear compartments, whereas in oPRL-treated animals the intensity of STAT5 fluorescence is markedly increased in the nucleus relative to the cytoplasm. *Scale bar*, 5 μm. B, The intensity of STAT5 immunostaining in the nucleus and cytoplasm was quantified in NIH image using the tyrosine hydroxylase profile to distinguish between the cellular compartments. Values are expressed as the ratio of STAT5 staining intensity in the nucleus to that in the cytoplasm (N/C ratio). For each animal, 30 neurons were quantified and results are the mean ± SEM for four rats per treatment group. *, $P < 0.05$.

The intensity of CIS immunofluorescence in TH-positive neurons in the dmArc was also quantified. Again, the TH (FITC) staining was used to delineate the cytoplasm and nuclear profile of individual TIDA neurons. For each animal, the mean intensity of CIS immunostaining (Texas Red) was quantified in the cytoplasm and nucleus from at least 15 TH-positive neurons in the dmArc. An estimate of background (Texas Red) staining was determined for each image, using an area close to the neuron that appeared to have no specific staining. This background measure was subtracted from the signal intensity of each compartment.

Northern analysis

Hypothalamic tissue was obtained from diestrous rats, continuously suckled lactating rats, and lactating rats 16 h after pup removal. Rats were killed by pentobarbitone. The brain was quickly removed and the hypothalamus dissected with a scalpel blade, frozen on solid CO_2 and stored at −80 C. Total RNA was extracted from hypothalamic tissue using Trizol reagent. Northern blot analysis for SOCS1, SOCS2, SOCS3, and CIS was performed as previously described (20, 21). Membranes were stripped and rehybridized with a probe to 18S rRNA for standardization. Densitometer scans were performed and results were expressed as fold induction relative to diestrous animals.

RT-PCR

The arcuate nucleus from diestrous and lactating rats with pups (n = 4 per group) was microdissected from thick (300 μm) coronal brain sections using a micropunch technique as previously described (22). Arcuate tissue punches were placed in Trizol reagent and the tissue disrupted mechanically by sonication. Total RNA was extracted and reverse transcribed as previously described (18) using the Superscript Preamplification System according to the manufacturers instructions (Life Technologies, Inc.). The RT product was amplified in PCR with specific rodent CIS forward (CTGGCTCCTTTCTTCTTCCG) and reverse (CACAAGGCTGACCACATCTG) primers designed from GenBank sequences (AJ243907 and AF065161). PCR was performed using 0.4 μl RT product, 0.5 μM of each primer, 0.2 mM deoxynucleotide triphosphate, 2 mM $MgCl_2$ and 1.3 U *Taq* DNA polymerase in 50 μl 1× *Taq* reaction buffer for 35 cycles (94 C, 30 sec; 61 C, 30 sec; 72 C, 30 sec). PCR products were visualized on an ethidium bromide-stained agarose gel. In addition, PCR with β-actin primers (forward, TGAACCCTAAGGCCAACCGTG; reverse GCTCATAGCTCTTCTCCAGGG) was performed for 24 cycles (94 C for 30 sec; 68 C for 30 sec; 72 C for 30 sec) as a positive control for each tissue sample.

Statistics

Data were analyzed using one or two-way ANOVA, followed by Student-Newman-Keuls *post hoc* test where appropriate. Data were log transformed before analysis to reduce heterogeneity of variance.

Results

PRL signals via STAT5 in TIDA neurons of diestrous rats

In this experiment, we used immunohistochemistry to confirm that PRL signals through STAT5 in the TIDA neurons of diestrous rats. Representative confocal images of TIDA neurons in the dmArc (Fig. 1A) show that in vehicle-treated rats STAT5 immunostaining in the nucleus is of similar intensity to that observed in the cytoplasm, whereas oPRL treatment markedly increases the STAT5 staining in-

tensity in the nucleus relative to the cytoplasm, indicative of STAT5 nuclear translocation. For each rat (n = 4 per treatment), the ratio of nuclear to cytoplasmic STAT5 immunostaining intensity (N/C ratio) was quantified for 30 TIDA neurons. Results revealed that oPRL injection significantly ($P < 0.05$, compared with vehicle-treated controls) increased the STAT5 N/C ratio in TIDA neurons (Fig. 1B).

PRL signaling via STAT5 is reduced in the TIDA neurons of continuously suckled rats

PRL negative feedback is reduced during lactation by the sucking stimulus. Here, we examined STAT5 signaling in the TIDA neurons of lactating animals in response to PRL injection. In lactating rats receiving vehicle injection, the N/C ratio of Stat5 immunostaining in TIDA neurons in the dmArc was similar in the presence or absence (16 h removal) of sucking pups (Fig. 2). However, in response to oPRL injection, there was a significant increase ($P < 0.05$) in the STAT5 N/C ratio in TIDA neurons of lactating rats that were deprived of their pups for 16 h. In contrast, oPRL injection had no effect on the STAT5 distribution in TIDA neurons of continuously suckled rats.

To demonstrate that responses in the TIDA neurons were specific to this dopaminergic cell group, we also quantified STAT5 immunostaining in the A13 dopaminergic neurons in the zona incerta, visible on the same coronal sections as the TIDA neurons above, and known not to express PRL receptors. In these neurons, no significant effect of oPRL injection was observed on the STAT5 N/C ratio in either pup-deprived (vehicle 1.07 ± 0.03, oPRL 1.00 ± 0.02), continuously suckled rats (vehicle 1.06 ± 0.03, oPRL 1.05 ± 0.01), or diestrous animals (vehicle 0.82 ± 0.02, oPRL 0.80 ± 0.03).

Is the resistance to PRL signaling through STAT5 dependent upon endogenous PRL levels?

Because the continuously suckled lactating rats in the previous experiment (Fig. 2) would have high endogenous PRL levels compared with animals with their pups removed, it is possible that the difference in PRL concentrations *per se* could account for the different STAT5 responses to injected PRL. We therefore treated animals with bromocriptine to suppress endogenous PRL and reexamined the STAT5 response to PRL injection. In continuously suckled lactating rats pretreated with bromocriptine, oPRL injection had no significant effect on the STAT5 N/C ratio in TIDA neurons (Fig. 3). In comparison, oPRL injection significantly ($P < 0.05$) increased the STAT5 N/C ratio in diestrous rats, despite pretreatment with bromocriptine (Fig. 3).

Are SOCS mRNAs up-regulated in the hypothalamus of lactating rats?

We hypothesized that diminished STAT5 responses observed in the TIDA neurons of lactating rats in the presence of sucking pups could be due to increased expression of a SOCS protein known to inhibit PRL signal transduction through STAT5. Accordingly, we performed Northern blot analysis for SOCS1–3 and CIS in the hypothalamus. The expression of SOCS1 and SOCS3 mRNAs in the hypothalamus were low (Fig. 4), and SOCS2 signals were undetectable. However, CIS mRNA was more readily detected and was significantly ($P < 0.01$) increased by 2-fold in continuously suckled lactating rats compared with both diestrous rats and lactating rats deprived of their pups. In contrast, SOCS1 and SOCS3 mRNA expression was not increased in lactation in response to sucking pups. Results shown in Fig. 4A are representative as experiments were replicated on many other tissue sets.

Furthermore, we examined whether increased hypothalamic CIS mRNA expression in lactating rats with pups was

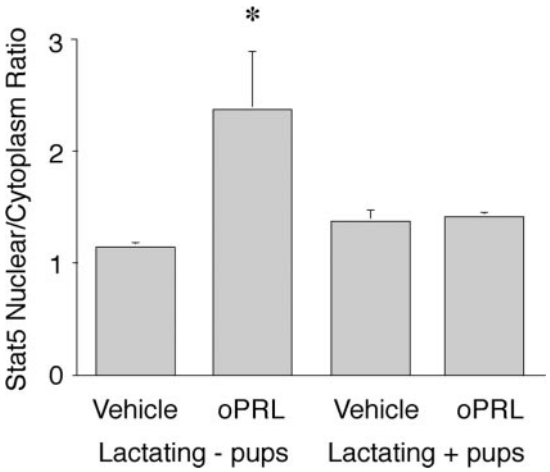

FIG. 2. STAT5 nuclear/cytoplasmic ratio in TIDA neurons of lactating rats in response to sc injection of either vehicle or oPRL (250 μg), given 1 h before death. Lactating rats either had their pups removed for 16 h (Lactating − pups) or were continuously suckled (Lactating + pups). Values are expressed as the ratio of STAT5 immunostaining intensity in the nucleus to cytoplasm. For each animal, 30 neurons were quantified and results are the mean ± SEM for four rats per treatment group. *, $P < 0.05$.

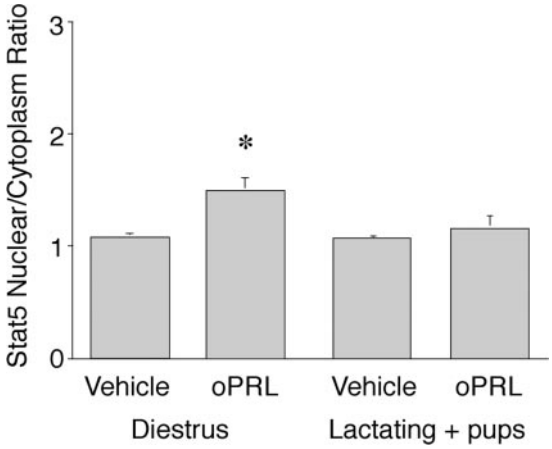

FIG. 3. STAT5 nuclear/cytoplasmic ratio in TIDA neurons of diestrous and continuously suckled lactating (Lactating + pups) rats treated with bromocriptine mesylate (3 mg/kg) for 2 h, before a sc injection of either vehicle or oPRL (250 μg) given 1 h before death. Values are expressed as the ratio of STAT5 immunostaining intensity in the nucleus to cytoplasm. For each animal, 30 neurons were quantified and results are the mean ± SEM for four rats per treatment group. *, $P < 0.05$.

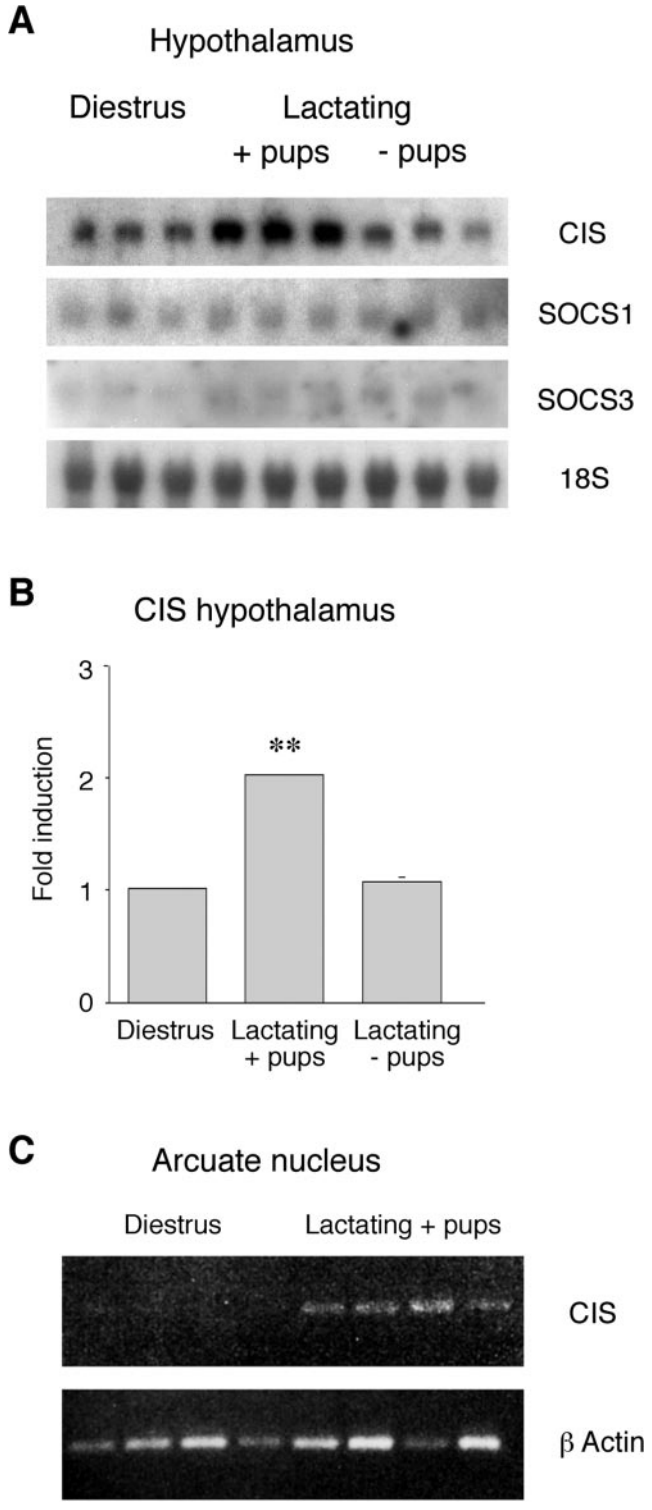

FIG. 4. A, Northern blot analysis of SOCS and CIS mRNA expression in the hypothalamus of diestrous and lactating rats. Lactating rats either had their pups removed for 16 h (Lactating − pups) or were continuously suckled (Lactating + pups). Total RNA was extracted from rat hypothalamic tissue and Northern blot was performed with 20 μg RNA per lane. Blots were stripped and reprobed for 18s to ensure equal loading. B, Quantitation of hypothalamic CIS mRNA. Results (mean ± SEM, n = 3/treatment) are expressed as fold induction relative to diestrus, after being normalized for 18s rRNA levels. **, $P < 0.01$. C, RT-PCR of arcuate nucleus micropunches with primers for CIS (*top panel*) and β-actin (*lower panel*). PCR products were electrophoresed on a 1.5% agarose gel and visualized with ethidium bromide. Each lane represents a different animal.

localized to the arcuate nucleus using a micropunch technique. Results from semiquantitative RT-PCR (Fig. 4C) show that CIS mRNA signals in the arcuate nucleus micropunches of all suckling lactating rats were detectable, whereas CIS signals in diestrous rats were undetectable.

Is CIS protein up-regulated in TIDA neurons?

We used dual-label immunofluorescence to determine whether CIS or SOCS3 was localized to TIDA neurons. Figure 5 shows representative images of CIS and SOCS3 immunoreactivity in TH-positive neurons in the dmArc nucleus. All TH-positive neurons in the dmArc expressed CIS in both diestrous and lactating rats. In addition, there were many CIS-positive cells that were not TH positive in the dmArc nucleus (Fig. 5, A–D). Furthermore, CIS immunoreactive perikarya were observed in other nearby hypothalamic regions including the ventrolateral arcuate nucleus, ventromedial nucleus, dorsomedial hypothalamus, lateral hypothalamus, and zona incerta (not shown). The distribution of CIS in the dmArc and other hypothalamic regions was confirmed with another CIS antisera (gift from A. Yoshimura).

In contrast to CIS, we did not observe any colocalization of SOCS3 in TH-positive neurons in the dmArc in any diestrous or lactating animal (Fig. 5, E and F). This result was confirmed using two different primary antisera. However, strong SOCS3 immunoreactivity was observed in perikarya located in the dorsomedial hypothalamus (Fig. 5, G and H), ventrolateral arcuate nucleus, ventromedial nucleus, perifornical nucleus, and lateral hypothalamus (others not shown).

To investigate whether CIS expression is increased in the TIDA neurons of lactating rats, we quantified the intensity of CIS immunostaining from confocal images of TH-positive neurons in the dmArc. In continuously suckled lactating rats, the intensity of CIS staining in either the cytoplasm (Fig. 6A) or nucleus (Fig. 6B) was significantly ($P < 0.05$) greater than that observed in either pup-deprived lactating rats, or diestrous rats. In most neurons, the intensity of CIS immunostaining in the cytoplasm was greater than the nucleus (see Fig. 5, C and D).

Discussion

The loss of sensitivity to PRL negative feedback during lactation by the sucking stimulus is an important physiological response that is necessary to maintain hyperprolactinaemia in the mother. This study shows for the first time that PRL signaling in TIDA neurons via the transcription factor STAT5 is reduced in continuously suckled lactating rats. To elucidate the mechanisms involved in this effect, we have made the novel observation that the sucking stimulus increases hypothalamic mRNA expression of CIS, a member of the SOCS family of proteins, known to negatively regulate PRL signaling through Jak/STATs. Indeed, we have found that all TIDA neurons in the dorsomedial arcuate nucleus

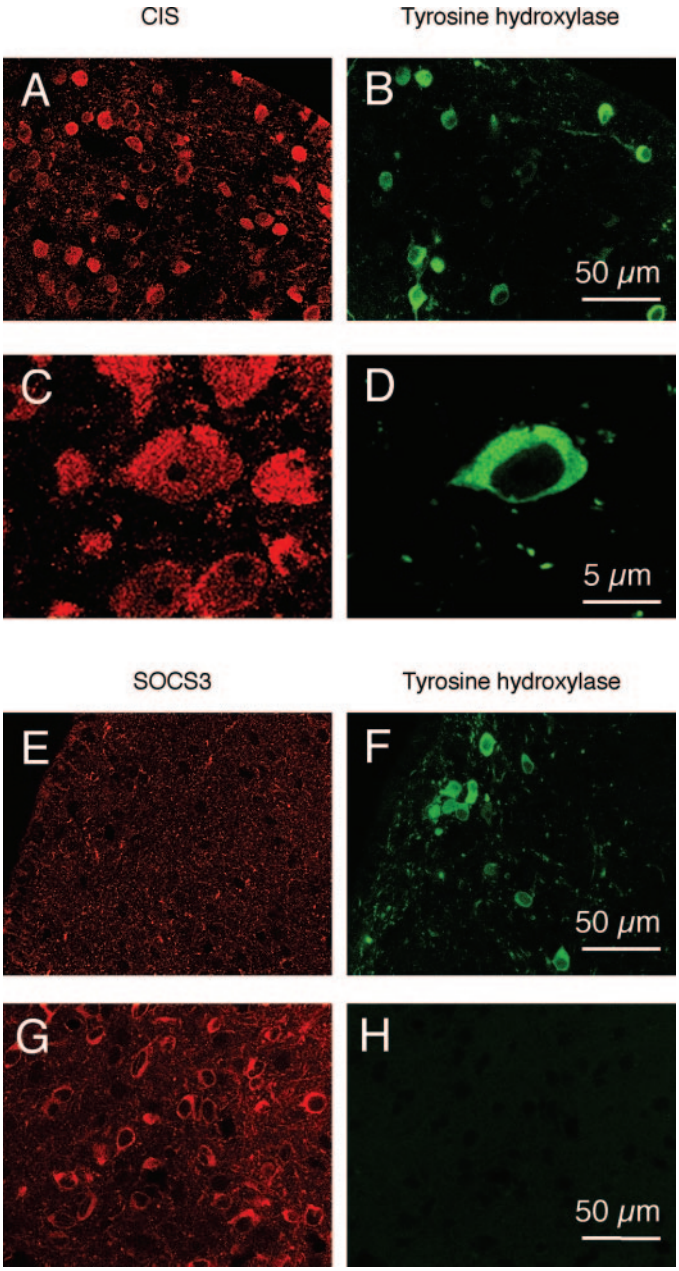

FIG. 5. CIS but not SOCS3 colocalizes with tyrosine hydroxylase in TIDA neurons. Representative confocal images of dual immunostaining for CIS or SOCS3 (*left column*, Texas Red stain) and tyrosine hydroxylase (*right column*, FITC stain) in the dmArc nucleus (A–F), and dorsomedial hypothalamus (G and H) of lactating rats.

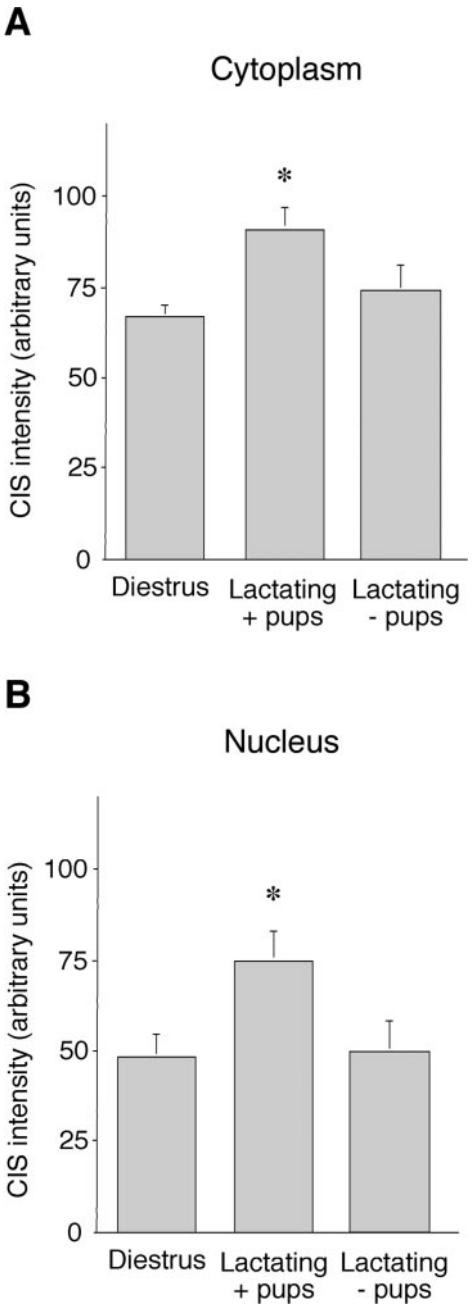

FIG. 6. Quantitation of CIS immunostaining in tyrosine hydroxylase-positive neurons in the dmArc of diestrous, continuously suckled lactating rats (Lactating + pups), and lactating rats that had their pups removed for 16 h (Lactating − pups). The intensity of CIS staining (Texas Red) was determined in the cytoplasm (A) and nucleus (B) of individual neurons using the tyrosine hydroxylase profile to distinguish between the compartments. For each animal, at least 15 neurons were quantified, and results are the mean ± SEM for four rats per treatment group. *, $P < 0.05$.

express CIS protein, and that CIS levels in TIDA neurons are increased in lactating rats in the presence of sucking pups.

Diminished responsiveness of the TIDA neurons to PRL negative feedback in lactation is well recognized (10–12, 23). Our results suggest that at least part of the reduction in negative feedback occurs at the PRL signal transduction level involving STAT5. PRL signals through various signal transduction pathways, and some involve STAT proteins, notably STAT1, STAT3, and STAT5 (24). However, the predominant signaling pathway for PRL in most cells appears to be via Jak2/STAT5. STAT5 has been previously localized to the TIDA neurons in the arcuate nucleus (8, 25). Indeed, STAT5 nuclear translocation in response to PRL in TIDA neurons had been noted in ovariectomized rats (8), and now we show similar effects in both diestrous and lactating rats where pups have been removed for 16 h. Moreover, the STAT5b isoform appears to be essential for normal PRL negative feedback because STAT5b-deficient mice have abnormally high serum

PRL concentrations due to a lack of negative feedback on TIDA neurons (9). Our results in lactating rats extend these findings, indicating that lactational hyperprolactinaemia due to the sucking stimulus is associated with a reduction of PRL-induced STAT5 signaling within the TIDA neurons.

There are a number of possible explanations for reduced PRL feedback via STAT5 in the TIDA neurons of lactating rats. For example, the lack of PRL signal transduction via STAT5 may be simply due to PRL receptor down-regulation. This appears unlikely because the numbers of neurons in the arcuate nucleus expressing immunoreactive PRL receptors are increased during lactation (13), and recent *in situ* hybridization studies (Kokay, I., and D. Grattan, personal communication) show that PRL receptor mRNA expression on TIDA neurons does not change in lactation. Another possible explanation for reduced STAT5 nuclear translocation is that there is increased expression of an inhibitor of PRL signaling that causes PRL resistance within the TIDA neurons. In this regard, the SOCS family of proteins are likely candidates because SOCS1, SOCS3, and CIS are well known to inhibit PRL signaling through Jak2/STAT5 (14, 14, 26, 27).

Our initial approach using Northern blots on whole hypothalamus suggested that CIS could be the factor causing resistance to PRL negative feedback in lactation. We observed increased CIS mRNA expression in the hypothalamus of continuously suckled lactating rats, compared with both diestrous and lactating rats with their pups removed for 16 h. Furthermore, in continuously suckled lactating rats we localized increased CIS mRNA expression to the arcuate nucleus using discrete micropunches and found significantly higher levels of CIS immunostaining within the TIDA neurons. Indeed, using two different CIS antisera we observed that all TH-immunoreactive neurons in the dmArc were immunoreactive for CIS. Together, these results strongly support the notion that loss of sensitivity to PRL negative feedback during lactation is a result of increased CIS expression in TIDA neurons. In contrast, our results do not suggest such a role for the other SOCS proteins that inhibit PRL signaling. We did not observe any increase in hypothalamic mRNA expression of SOCS1 and SOCS3 in lactating rats with pups, whereas SOCS2 was undetectable. However, because our Northern blots were performed on whole hypothalamus, they may not be indicative of changes in SOCS expression within TIDA neurons, so SOCS1–3 cannot be excluded. Nevertheless, SOCS3 is unlikely to be important in PRL negative feedback because we did not observe SOCS3 immunoreactivity in the TIDA neurons of lactating (or diestrous) rats. So overall, our studies suggest that increased CIS expression in the TIDA neurons contributes to the loss of sensitivity to PRL negative feedback during lactation, although involvement of SOCS1 and SOCS2 cannot be excluded.

Despite strong *in vitro* evidence that CIS inhibits PRL signaling through STAT5 by binding directly to the PRL receptor (28, 29), few *in vivo* studies have investigated physiological roles for CIS. Mice overexpressing CIS display reduced STAT5 phosphorylation in the mammary glands and fail to lactate after parturition (26), indicative of a failure in PRL signal transduction. In contrast, CIS null mice do not display any overt phenotype (30). However, to our knowledge circulating PRL concentrations have not been examined in either of these animal models. As a logical extension of our present results, we would predict the CIS null mouse to have abnormally low PRL levels during lactation. The fact that these animals lactate normally (Ihle, J., personal communication) could indicate a redundant role for CIS in the TIDA neurons. Alternatively, PRL levels in CIS null mice may be reduced but still sufficient to stimulate lactogenesis, and only close examination of PRL levels in these animals would reveal any deficit.

Whether the increase in CIS expression is a consequence of high endogenous PRL levels caused by the sucking stimulus or a consequence of other mechanisms remains to be determined. Up-regulation of SOCS/CIS expression in cells to date has been viewed primarily as direct consequence of cytokine stimulation, with increased SOCS/CIS expression preventing further stimulation by the cytokine and therefore cellular resistance. However, increasingly other signaling molecules, for example angiotensin (31), TSH (32), and prostaglandin F2α (21), have been shown to modulate expression of SOCS proteins and therefore could induce cytokine resistance. In relation to the TIDA neurons, it is worth noting from our results that bromocriptine treatment, to suppress endogenous PRL levels, did not modify the lack of STAT5 responsiveness to PRL in the TIDA neurons of lactating rats (Fig. 3). Also, we did not observe any change in hypothalamic CIS mRNA expression in lactating rats after bromocriptine treatment (see the supplemental data published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). Certainly, the return of sucking pups to their mothers after 16 h of separation markedly increases hypothalamic CIS mRNA expression within 1 h (see supplemental data). Therefore, it is likely that the increased CIS expression in lactating rats in response to the sucking stimulus is a consequence of stimuli other than PRL, possibly neuronal inputs to the TIDA neurons, and this warrants further investigation.

In conclusion, our results indicate that PRL negative feedback on TIDA neurons involves nuclear translocation of STAT5 and this is disrupted in lactating rats by the sucking stimulus. We have further shown that CIS, a member of the SOCS family of proteins known to negatively regulate PRL signaling, is up-regulated in the hypothalamus during lactation by the sucking stimulus. Moreover, CIS protein is localized to TIDA neurons that mediate PRL negative feedback, and in lactating rats CIS levels in these neurons is increased in the presence of sucking pups. Together, the results support the hypothesis that loss of sensitivity to PRL negative feedback during lactation is a result of increased expression of CIS in the TIDA neurons.

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