Passive immunization of lactating mice with stanniocalcin-1 antiserum reduces mammary gland development, milk fat content, and postnatal pup growth

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Zaidi, Deenaz, Kathi A. James, and Graham F. Wagner. Passive immunization of lactating mice with stanniocalcin-1 antiserum reduces mammary gland development, milk fat content, and postnatal pup growth. Am J Physiol Endocrinol Metab 291: E974–E981, 2006.—During pregnancy and lactation in rodents, stanniocalcin-1 (STC-1) production by the ovaries is upregulated markedly and released into the circulation. The mammary glands are one target of this systemically delivered hormone. The purpose of this study was to lower serum levels of STC-1 in lactating mice through passive immunization so as to monitor the effects on mammary gland function and postnatal pup growth. Passive immunization significantly reduced circulating hormone levels, and pup growth was significantly compromised (30%), even though control and experimental litters had ingested equal amounts of milk. When mammary glands were analyzed, the alveolar area was significantly reduced in antibody-treated mothers. An analysis of milk composition revealed no changes in lactose, protein, or electrolyte levels but an increase in triglycerides. Locomotor effects suggest that STC-1 also has a role in mammary gland development, milk fat content, and postnatal pup growth, and maternal behavior.

MATERIALS AND METHODS

Reagents. Polyclonal antibodies to human (h)STC were generated in rabbits against recombinant human STC-1 (rhSTC-1) and have been characterized for specificity by Western blotting and radioimmunoassay. They show no known cross-reactivity to other hormones, such as LH, FSH, GH, PRL, or human chorionic gonadotropin (35). Normal rabbit serum (NRS) was obtained from Sigma.

Passive immunization of lactating mice. Four-week-old male and female CD-1 mice were obtained from Charles River laboratories and housed and bred in the Health Sciences Animal Care Facility at the University of Western Ontario on a 12:12-h light-dark cycle. At birth (day 1 of lactation), the number of pups was standardized to eight for each litter. All pups were numbered with a surgical marker for ease of identification, and mothers plus litters were arbitrarily divided into control and experimental groups. Experimental mothers were injected with STC antiserum, whereas control mothers received NRS. Injections were given twice daily at a dosage of 50 μl per injection (100 μl total per day) on days 1–8 of lactation. Injections were given subcutaneously in the morning (9:00 AM) and late afternoon (4:00 PM). The study was repeated twice more (3 times total).

Postnatal pup growth. The pups of both control and experimental mothers were weighed daily to determine whether antibody treatment had any effect on postnatal growth rate. Groups were then compared statistically by repeated-measures ANOVA. In addition to these daily recordings of weight, pups were weighed before and after nursing events. In the latter case, each group of pups was weighed before and after

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after four separate nursing events during the 8-day study. This was done to see whether antibody treatment had any effect on the weight of milk ingested. Groups were compared statistically with a paired Student’s t-test.

Milk collection and mammary gland tissue preparation. On day 8 of lactation, the pups were separated from their mothers for 8—10 h to allow for milk buildup. During the separation period, the mothers received a final injection of NRS or STC antiserum as described above. Twenty minutes before milking, the mothers were injected with 5 IU/30 g body wt of oxytocin to induce milk letdown. The mothers were then anesthetized with 62.5 mg/kg body wt pentobarbital sodium. Milk from the anesthetized mothers was extracted by vacuum pulsation (14).

After milk extraction, the animal was euthanized, and blood was collected by cardiac puncture and allowed to clot overnight at 4°C. The sera were stored at 70°C for subsequent analysis of STC-1 content by radioimmunoassay. Thoracic mammary glands were excised, frozen on dry ice, and stored at −70°C for further analysis. The inguinal and abdominal mammary glands were weighed and immersion fixed in fixative containing 4% paraformaldehyde (PFA), 0.01 M sodium phosphate, pH 7.4, and 0.15 M NaCl. The pups of each group were euthanized with CO2 and either frozen or immersed in PFA fixative.

Mammary gland morphometric analysis. The inguinal and abdominal mammary glands of control and experimental mothers were dehydrated and embedded and cast into parafin blocks for sectioning. Sections 5 μm thick were cut and stained with hematoxylin and eosin for histological analysis. Upon histological analysis, a clear difference was evident in alveolar size between the control and experimental mothers. Therefore, photomicrographs were taken for morphometric analysis using Image Pro Plus software to measure the alveolar area. The boundaries of randomly chosen alveoli (40 per group) were traced with a caliber pen to obtain alveolar area in arbitrary units. Mean alveolar areas were then statistically analyzed by an unpaired two-tailed Student’s t-test. Additional sections were treated with Masson’s trichrome stain to visualize nuclei, cells, and the connective tissue compartment.

Mammary gland receptor-binding studies. To observe any effects of passive immunization on STC-1 receptor levels, the paired thoracic mammary glands in each group were pooled (n = 5) and subjected to Dounce homogenization to isolate the nuclear, mitochondrial, and microsomal membrane fractions. Receptor-binding studies were then carried out on each fraction to obtain estimates of Kd and Bmax, as previously described (16). Values obtained were analyzed statistically with an unpaired two-tailed Student’s t-test.

Milk, serum, and fecal analyses. Microscopic analysis of the alveolar lumen revealed a visible reduction in the number of milk fat droplets in antibody-treated mothers (data not shown). This prompted an analysis of milk fat content. Initially this was done using the crematocrit method (30). On our observing a reduction in milk fat content of antibody-treated mothers by this method, triglyceride assays were performed, using a commercial kit (Roche Diagnostics, Indianapolis, IN), to more accurately assess the change. Blood triglyceride levels were also assessed, with the same assay, on triplicate 10-μl aliquots of serum from control and experimental mothers. Triglyceride concentrations were expressed in milligrams per 100 milliliters and compared with an unpaired two-tailed Student’s t-test. Milk levels of calcium, phosphate, potassium, and magnesium were assessed by atomic absorption spectrophotometry (London Health Sciences Centre, London, ON, Canada), expressed in milligrams per gram of milk and compared with an unpaired two-tailed Student’s t-test.

A double-antibody radioimmunoassay (RIA) was used to assess the changes in maternal levels of serum STC-1 after 8 days of passive immunization (35). The assay has already been characterized for STC-1. Prior to analysis and to avoid any possible interference in the RIA, residual rabbit IgG (from the last injections of NRS and STC-1 antiserum) was cleared from the serum by immunoprecipitation. This involved adding NRS and goat anti-rabbit IgG (in equivalence) to each serum sample, followed by an overnight incubation on ice. The sera were then centrifuged for 10 min at 12,000 g to pellet the resulting immune complexes and recover the supernatants. Triplicate 100-μl aliquots of the resulting supernatants were then assayed for STC-1 content. Groups were compared with an unpaired two-tailed Student’s t-test.

The feces were extracted from individual control and experimental pups, and total fecal triglyceride levels were measured using a commercial kit (Wako Chemicals, Neuss, Germany), as previously described (29). Groups were compared with an unpaired two-tailed Student’s t-test.

Mammary gland lipoprotein lipase activity. An enzymatic assay was used to measure lipoprotein lipase (LPL) activity in control and experimental mammary glands (22). The assay works on the principle that the LPL present in the sample acts on a triglyceride substrate (Intralipid, a soybean triglyceride substrate) to liberate free fatty acids. The resulting free fatty acid levels are indicative of LPL activity of the sample. Free fatty acids were determined using a commercial kit (Wako Chemicals). LPL activity was expressed in enzymatic units where one unit of lipase activity was equal to 1 μmol free fatty acid liberated·h−1·mg protein−1. Groups were compared with an unpaired two-tailed Student’s t-test.

Pup body fat and bone mineral content analysis. Dual-energy X-ray absorptiometry (DEXA) analysis was used to measure the body fat and bone mineral content of pups from control and experimental mothers (2, 20, 31, 48), employing the services of the Centre for Modeling Human Disease (Mount Sinai Hospital, Toronto, ON, Canada). This analysis was done on pups fixed in 4% paraformaldehyde with the help of the PIXIMUS Small Animal Densitometer. The body fat and bone mineral content of each pup were expressed as a percentage of body weight and aincise transformed prior to statistical analysis with an unpaired, two-tailed Student’s t-test.

Behavioral studies on lactating mothers. On account of there being overt hyperactivity in antibody-treated mothers, locomotor activity and behavior were both analyzed by videotaping mothers with their pups to characterize any changes in behavior. Mothers plus litters were videotaped on three separate days (25 min per session) during the 8 days of passive immunization. Sessions were conducted at mid-day (noon–2:00 PM) between injections, and the order of filming (controls vs. experimental groups) was randomized. Behavioral characteristics were categorized into movement types that included cage running (one complete run defined as traveling from the end of the cage, where the nest was placed, to the other end and back again), rearing on hindlegs, self-grooming, and scratching the base of the nest. Behavior was analyzed as above in three control and three antibody-treated mothers. Groups were compared with an unpaired two-tailed Student’s t-test.

RESULTS

Effects of passive immunization on maternal serum STC-1 levels and mammary gland receptor levels. The success of passive immunization was revealed by RIA analysis of serum STC-1 (Fig. 1). Hormone levels were significantly lower in antibody-treated mothers vs. the controls (P < 0.005, Student’s t-test). Similar reductions in serum STC-1 levels were obtained in the two other studies. Receptor-binding studies revealed that membrane levels of STC-1 receptors were upregulated to a small but statistically significant degree as a result of passive immunization (P < 0.05, Student’s t-test; Table 1), whereas those in the nuclear and mitochondrial fractions were unchanged. Receptor affinity (Kd) was reduced on both membranes and nuclei (P < 0.05, Student’s t-test; Table 1) but unchanged in mitochondria.
Effects of passive immunization on postnatal pup growth. Figure 2 shows the growth curves of pups from NRS and STC-1 antibody-treated mothers. Over the 8-day study, repeated-measures ANOVA revealed that experimental pups suffered a significant decrease in weight gain, commencing after 3 days of treatment, and weighed 30% less after 8 days of treatment (P < 0.0001). Similar effects on growth were obtained in the two other studies. In contrast, antibody treatment had no effect on maternal growth after 8 days of passive immunization (57.8 ± 3.3 g in antibody-treated mothers vs. 58.2 ± 3.4 g in controls, means ± SE, n = 5, P > 0.05, Student’s t-test).

Because the reduction in pup weight might be attributable to changes in the amount of milk ingested, all pups were weighed before and after three separate nursing events, and the percent changes in the amount of milk ingested, all pups were weighed. Figure 4 shows that pups from antibody-treated mothers (Fig. 3, A and B, insets). A weight comparison of inguinal and abdominal mammary glands revealed no differences between treatment groups (0.12 ± 0.1 vs. 0.11 ± 0.1 g in controls; n = 5, P > 0.05, Student’s t-test). Masson’s trichrome staining also revealed no overt differences in the connective tissue compartment between treatment groups (results not shown).

Mammary gland morphometric analysis. Microscopic observations revealed a marked reduction in alveolar area in antibody-treated mothers (Fig. 3, A and B). Morphometric analysis was performed to quantify the differences (Fig. 3C) and revealed that alveolar area was significantly reduced by antibody treatment (P < 0.001, Student’s t-test). Statistically different reductions in alveolar diameter were likewise obtained in the other two studies. There were no overt differences between groups, however, in the appearance of the alveolar cells (Fig. 3, C and D). Morphometric analysis revealed no differences between treatment groups (0.12 ± 0.1 vs. 0.11 ± 0.1 g in controls; n = 5, P > 0.05, Student’s t-test).

To determine whether the reduction in milk triglycerides resulted in a reciprocal buildup in the serum, serum triglycerides were analyzed in control and antibody-treated mothers. Figure 4B shows that serum triglyceride levels were increased by 50% in antibody-treated mice (P < 0.005, Student’s t-test). Similar effects were observed in the two other studies.

The low triglyceride levels in milk from antibody-treated mothers, coupled with its buildup in the serum, prompted an examination of mammary gland LPL activity, the principal enzyme responsible for the cleavage of serum-based triglycerides (Fig. 4C). This revealed a marked reduction (~46%) in enzyme activity following antibody treatment (P < 0.0001, Student’s t-test). Similar effects were observed in all three studies.

The analysis of fecal triglycerides (Fig. 4D) revealed that they were twice as high in pups from antibody-treated mothers compared with controls (P = 0.02, Student’s t-test). Similar

Table 1. Receptor-binding studies on mammary gland subcellular fractions

<table>
<thead>
<tr>
<th>Subcellular Fraction</th>
<th>NRS</th>
<th>STC Antibody</th>
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<tbody>
<tr>
<td>Membrane Kd</td>
<td>1.35±0.28</td>
<td>3.46±0.73*</td>
</tr>
<tr>
<td>Membrane Bmax</td>
<td>10.61±0.88</td>
<td>14.63±1.65*</td>
</tr>
<tr>
<td>Nuclear Kd</td>
<td>0.70±0.19</td>
<td>1.5±0.28*</td>
</tr>
<tr>
<td>Nuclear Bmax</td>
<td>15.49±1.45</td>
<td>18.61±1.39</td>
</tr>
<tr>
<td>Mitochondrial Kd</td>
<td>1.59±0.48</td>
<td>1.99±0.61</td>
</tr>
<tr>
<td>Mitochondrial Bmax</td>
<td>10.5±1.3</td>
<td>14.2±1.8</td>
</tr>
</tbody>
</table>

Kd (nM) and Bmax values (pmol/mg) are shown as means ± SE; n = 22.

NRS, normal rabbit serum; STC, stanniocalcin. *Significantly different from NRS controls (P < 0.05, 2-tailed Student’s t-test).
effects were seen in one other study in which fecal fat levels were examined.

**Pup body fat content and bone mass.** The differences in postnatal pup growth rates prompted a comparison of body fat and bone mass content by DEXA analysis. This revealed a small, albeit significant, decrease in body fat content in pups from passively immunized mothers (14.6 ± 0.3 vs. 17.1 ± 1.0% in controls; \( n = 5, P < 0.05 \), Student’s t-test). Thus the decline in growth rate was attributable, in part, to a reduction in body fat content. In contrast, there were no differences in bone mass (29.6 ± 6.8 vs. 36.4 ± 6.0% in controls; \( n = 5, P > 0.05 \), Student’s t-test).

**Maternal behavioral studies.** Videotape monitoring of control and passively immunized mothers revealed significant differences in behavior in terms of both cage running and hindleg rearing (Fig. 5). Cage running occurred five times more often in antibody-treated mothers compared to NRS-treated controls (Fig. 5).}

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**Fig. 3.** Mammary gland histology and morphometric analysis. Histological analysis revealed marked differences in the size of individual alveoli (arrows) between mothers injected with STC antiserum (A) or NRS (B). However, there were no overt differences in the appearance of alveolar cells between treatment groups (insets). C: morphometric analysis revealed that alveolar area was significantly reduced by treatment with STC antiserum (\( n = 4, \text{means} \pm \text{SE}; ***P < 0.001, \text{Student’s t-test} \)).

**Fig. 4.** Parameters of fat metabolism in mothers and pups. A: treatment with STC antiserum resulted in a 38% reduction in milk triglycerides vs. NRS-treated controls (\( n = 5; ***P < 0.001, \text{Student’s t-test, means} \pm \text{SE} \)). B: treatment with STC antiserum also caused a buildup of serum triglycerides (\( n = 5, \text{means} \pm \text{SE}; ***P < 0.005, \text{Student’s t-test} \)). C: maternal mammary gland lipoprotein lipase (LPL) activity was significantly reduced by antibody treatment vs. NRS-treated controls (\( n = 5, \text{means} \pm \text{SE}; ***P < 0.0001, \text{Student’s t-test} \)). One unit of lipase activity was equal to 1 µmol free fatty acid liberated h \(^{-1} \) mg protein \(^{-1} \). D: steatorrhea was evident in pups from antibody-treated mothers vs. NRS-treated controls, indicative of malabsorption of intestinal fat (\( n = 5, \text{means} \pm \text{SE}; *P = 0.02, \text{Student’s t-test} \)).
tumors and preneoplastic ductal hyperplasia (52). Treatment with inhibin antiserum can induce superovulation in mice (27). In the context of the present study, antibodies to fish STC-1 effectively neutralize the circulating hormone in fish and in doing so reverse its inhibitory effects on gill calcium transport (57). The present study has now shown that passive immunization with antibodies to hSTC-1 can suppress serum STC-1 levels in lactating mice to the extent that membrane levels of mammary gland STC-1 receptors are upregulated, likely due to reduced ligand targeting. More importantly, the suppression of serum STC-1 had pronounced effects on mammary gland development, milk fat content, and postnatal pup growth. Overt effects on maternal behavior were observed as well. As such, the results provide functional evidence as to the purpose for systemic targeting of STC-1 to the rodent mammary gland during lactation.

Mammary gland development. Histological and morphometric analyses revealed a marked reduction in alveolar area in antibody-treated mothers. Mammary gland terminal differentiation occurs during late pregnancy (lactogenesis I) and during lactation (lactogenesis II) and is necessary to achieve full lactational capacity (32). Because suppressing the hormone so clearly affected lactogenesis II, STC-1 would appear to be implicated in mammary development, along with progesterone and PRL, the principal known regulators of mammary development (18, 26, 33, 36).

Progesterone is essential during lactogenesis I for ductal side-branching and alveologenesis, acting via receptors on stromal and epithelial cells (5, 19, 26). Serum progesterone levels then decline 2–3 days before parturition and are low during lactation, when serum levels of STC-1 are correspondingly elevated (10). As STC-1 is a known inhibitor of luteal cell progesterone release (38), it could very well be involved in suppressing progesterone levels during the lactation phase. Consequently, if progesterone levels were allowed to rise in the present study due to the suppression of serum STC-1, this, in combination with the reduction in STC-1 targeting, may have contributed to the reduction in alveolar size.

PRL stimulates ductal development during early pregnancy, as well as lobuloalveolar differentiation during late pregnancy and lactation (5, 56). Mammary gland PRL receptor levels are low in mice early in pregnancy but rise later on (28, 34) and are abundant on alveolar cells during lactation. The long PRL receptor isoform is involved in lobuloalveolar differentiation (28, 34), mediated via the STAT5a pathway. STAT5a−/− mice fail to develop functional mammary glands and are incapable of lactating (25). Similarly, mammary gland growth is arrested at the stage of extended ductal outgrowths in PRL−/− and PRL receptor−/− mice (24). It is also recognized that some of PRL’s effects are indirect and mediated through the actions of other hormones (4). A relationship between STC-1 and PRL has yet to be established in mammals; however, they both regulate gill Ca2+ transport in fish (57) and could very well interact in the regulation of lactogenesis II, especially as the secretion of each is so highly dependent on the suckling stimulus.

Changes in mammary gland morphology also entail extensive remodeling of the extracellular matrix (49), involving cell adhesion molecules and matrix-remodeling enzymes such as stromelysin (54, 60) and E- and P-cadherin (9). However, Mason’s trichome staining revealed no marked differences in mammary gland collagen content. Hence, the observed devel-

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**DISCUSSION**

Passive immunization has a long history of use in defining the actions of peptide hormones. Antibodies to neuropeptide Y have been used to reveal its role in the secretion of gonadotropins and prolactin (PRL) (13), whereas PRL receptor antibodies have helped delineate the role of PRL ligand in lactogenesis I, the gestational stage of mammary gland development (52). As a potential therapeutic, PRL receptor antibodies also decrease the occurrence of PRL-dependent mammary gland

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**A**

Cage Runs / Session

NRS STC Antiserum

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**B**

Rearing / Session

NRS STC Antiserum

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**C**

Grooming / Session

NRS STC Antiserum

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Fig. 5. Behavioral activity in control and passively immunized mice. A: cage running. Treatment with STC antiserum produced a 5-fold increase in cage running activity vs. NRS controls (n = 3; ***, P < 0.005, Student’s t-test). B: hindleg rearing activity. Treatment with STC antiserum produced a 4-fold increase in hindleg rearing vs. NRS controls (n = 3; ***, P < 0.005, Student’s t-test). C: self-grooming. There were no differences in grooming frequency (n = 3; P > 0.05, Student’s t-test).
intestinal fat absorption. If milk STC-1 levels were indeed derived STC-1 to milk and, in doing so, negatively impacted passive immunization may have reduced the transfer of serum-governed fat absorption by the neonate gut. In this regard, therefore be indicative of an endocrine-exocrine pathway that derived, as STC-1 gene expression is all but undetectable in the and immunocytochemistry, most of which is likely serum contains measurable amounts of STC-1, as revealed by RIA (8) treated pups, indicative of intestinal malabsorption. Milk con-

The results have shown that, compared with NRS-injected controls, antibody-treated pups had significantly reduced levels of body fat. A significant proportion of this can be attributed to the low triglyceride levels in the milk. However, there were also significantly more triglycerides in the feces of antibody-treated pups, indicative of intestinal malabsorption. Milk contains measurable amounts of STC-1, as revealed by RIA (8) and immunocytochemistry, most of which is likely serum derived, as STC-1 gene expression is all but undetectable in the lactating mammary gland (16). Its presence in milk may therefore be indicative of an endocrine-exocrine pathway that governs fat absorption by the neonate gut. In this regard, passive immunization may have reduced the transfer of serum-derived STC-1 to milk and, in doing so, negatively impacted intestinal fat absorption. If milk STC-1 levels were indeed reduced by antibody treatment, this could have negatively affected those enzymes that are responsible for the intestinal absorption of fat. Precedents for the presence of peptide hormones in milk have already been established in the case of parathyroid hormone-related peptide (PTHrP) (7, 45) and leptin (11). PTHrP is believed to have positive absorptive effects on the neonate gut by relaxing visceral smooth muscle and increasing luminal gut volume (41). It has also been suggested that ingested PTHrP may enter the neonate circulation for the regulation of calcium homeostasis (17, 47). Leptin is also secreted into milk (under the influence of PRL), where it augments milk fatty acid synthesis (11). Given these prece- dents, it is not unreasonable to suppose that milk-based STC-1 could also have regulatory effects on the neonate gut.

Maternal behavior. Various behaviors are exhibited by lactating mice, including locomotor activity, nursing, pup grooming, nest repair, and pup retrieval (43, 53). Of these, the most striking differences observed in antibody-injected mothers were hyperactivity, manifested by constant cage running, and hindleg rearing. However, as pup retrieval was unchanged and both groups nursed equally well, the hyperactivity did not affect feeding.

Hindleg rearing has been attributed to anxiety and stress (55), and, interestingly, there is a direct relationship between dietary fat and the stress response. In rat pups, high levels of milk fat have been found to reduce their response to stress. Moreover, the effect is mediated by leptin (58), thus implying a relationship among the stress response, dietary fat, and leptin. Leptin has also been associated with increased locomotor activity. In genetically obese, leptin-deficient (ob/ob) mice, leptin treatment not only decreases food intake but also increases locomotor activity (40). Also, adipocyte leptin release is suppressed during lactation to permit continuous maternal feeding (6, 23). As adipocytes also contain high levels of STC-1 receptors (37), it is possible that STC-1 is involved in this suppression and that passive immunization led to an increase in serum leptin levels and the consequent behavioral effects.

Conclusions. Our results have shown that the immunoneutralization of STC-1 in lactating mice has pronounced effects on mammary gland development, LPL activity, postnatal pup growth, and maternal behavior. The study leaves many questions unanswered, perhaps the most important of which is whether or not these effects were direct or were mediated through the actions of hormones such as progesterone, prolactin, insulin, and leptin.

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