Effects of Active Immunization against Somatostatin or its Analogues on Milk Protein Synthesis of Rat Mammary Gland Cells

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ABSTRACT : Effects of active immunization against native 14-mer somatostatin (SRIF, somatotropin releasing inhibiting factor) and its two 14-mer-somatostatin analogues on the milk production in rat mammary cells were studied. Native SRIF, Tyr11-somatostatin (Tyr11-SRIF), and D-Trp8, D-Cys14-somatostatin (Trp8Cys14-SRIF) were conjugated to bovine serum albumin (BSA) for immunogen preparation. Twenty-four female Sprague-Dawley rats were divided into four groups and immunized against saline (Control), SRIF, Tyr11-SRIF, and Trp8Cys14-SRIF at five weeks of age. Booster immunizations were performed at 7, 9, and 11 weeks of age. SRIF-immunized rats were mated at 10 weeks of age. The blood and mammary glands were collected at day 15 post-pregnancy and -lactation. To measure the amount of milk protein synthesis in the mammary gland, mammary cells isolated from the pregnant and the lactating rats, were cultured in the presence of ^{3}H-lysine. No significant differences in growth performance, concentration of growth hormone in the circulation, and the amount of milk protein synthesis were observed among the groups. Inductive levels of serum anti-SRIF antibody in the SRIF and Tyr11-SRIF groups but not in the Trp8Cys14-SRIF group, were significantly higher than that of the control group during the pregnancy and lactation periods. The result suggests that active immunization against native 14-mer SRIF and Tyr11-SRIF was able to induce anti-SRIF antibodies, but did not affect the milk protein synthesis. ***(Asian-Aust. J. Anim. Sci. 2002. Vol 15, No. 4 : 570-575)***

Key Words : Somatostatin, Active Immunization, GH Concentration, Milk Protein Synthesis

INTRODUCTION

Growth hormone (GH), a polypeptide hormone synthesized and secreted by the pituitary gland, is a potent stimulator not only for the growth of pre- and post-natal animals, but also for milk production in lactating cows (Bchini et al., 1991; Flint et al., 1992; Flint and Gardner, 1994; Travers et al., 1996; McMahon et al., 2001). Similar results can also be obtained through the administration of recombinant bovine GH (Baumon et al., 1985; Myung, 1990).

Two hypothalamic peptides [GH-releasing hormone (GHRH) and somatostatin (SRIF, somatotropin releasing inhibiting factor)] are responsible for controling the baseline level of GH and regulating the GH secretion from the pituitary gland (Liberti and Joshi, 1986; Stolar and Baumann, 1986; Sinha and Jacobsen, 1988). GHRH stimulates the release and magnitude of GH (Wehrenberg et al., 1982; Wehrenberg et al., 1983; Etherton et al., 1986; Yoyoka and Friesen, 1986; Painson and Tannenbaum, 1991) and is, therefore, responsible for enhancing the growth performance (Tannenbaum and Ling, 1984; Tannenbaum and Bowers, 2001). SRIF exerts diverse physiological actions in the body including regulation of hormone and neurotransmitter release (Tannenbaum and Ling, 1984; Yoyoka and Friesen, 1986; Painson and Tannenbaum, 1991). Analogs of SRIF are used clinically to treat tumors and cancers and to block the hyper-secretion of growth (Desai et al., 2001).

Immunoneutralization of SRIF has been suggested as a potential method for enhancing meat and milk production because SRIF is a potential inhibitor of GH secretion (Laarveld et al., 1986; Garssen et al., 1987). Passive immunization against SRIF elevated the levels of endogenous GH secretion (Chihara et al., 1983; Lanzi and Tannenbaum, 1992; Tannenbaum et al., 1992) and prevented inhibitory effect of GH secretion induced by stress (Arimura et al., 1976) and starvation (Tannenbaum et al., 1978). Active immunization against SRIF increased GH release in growing pigs (Dubreuil et al., 1989) and improved the growth rate of lambs by 13-34% (Laarveld et al., 1986; Mears, 1990, 1995). To the contrary, some studies have shown that the immunization against SRIF did not affect the growth rates in gilts (Du and Hacker, 1992, 1993) and steers (Machen et al., 1987; Dawson et al., 1997).

Effects of active immunization against SRIF during lactation also showed conflicting results. Positive effects on the milk production were reported by some groups (Garssen et al., 1987; Farmer and Brazeau, 1992), while no effect was revealed in a different study (Farmer et al., 1990). These inconsistent results may be due to differences in the induction level of antibodies depending on the antigen preparation, antigenic peptide, adjuvant, dosage, species,

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Received September 20, 2001; Accepted November 5, 2001

and age (Coligan et al., 1991).

Previously, we examined various forms of SRIF to test their ability to invoke antibody production against SRIF (Yi et al., 1999; Kim et al., 2001). Only native 14-mer SRIF induced anti-SRIF antibodies, which did not improve the growth rate and the milk production. We postulated that the antibody production through injection with 14-mer SRIF was not sufficient to induce positive effect on the growth performance and milk production. The objective of this study was thus to investigate the effects of active immunization against native 14-mer SRIF or two 14-mer SRIF analogues on the milk protein synthesis.

MATERIALS AND METHODS

Animals and diets

Twenty-four female Sprague-Dawley rats were obtained from Samyung laboratory animal center (Osan, Korea) and were individually housed at 12 h light and 12-h dark cycle. Temperature was maintained at 20±3°C. Animals were randomly assigned to one of the following four treatments: Control, SRIF, Tyr11-SRIF, and Trp8Cys14-SRIF. Six rats were allotted into each group and were fed *ad libitum* with free access to water. Daily feed intake was monitored individually and each animal was weighed weekly basis throughout the experiment. The formula and chemical composition of experimental diets are shown in table 1. Rats were mated at ten weeks of age, and pregnancy was confirmed using vaginal plugs.

Immunization and blood sample collection

SRIF, Tyr11-SRIF and Trp8Cys14-SRIF were purchased from Sigma (St. Louis, Mo, USA). As shown in table 2, Tyr11-SRIF and Trp8Cys14-SRIF had one amino acid substitution at position 11 and two at positions 8 and 14, respectively. Each antigen was conjugated to bovine serum albumin (BSA) using the glutaraldehyde-coupling method as described by Spencer et al. (1983). The conjugates were dialyzed in phosphate-buffered saline (PBS) and were kept at -20°C until use. Final concentration of the conjugates was 1.0 mg/ml. For the primary immunization, the antigen, conjugated to BSA in PBS mixed with Freund's complete adjuvant at a ratio of 1:2 (vol/vol), was given at 5 weeks of age. A total of 0.15 ml emulsion containing 150 μg of antigen was subcutaneously injected into both sides of the shoulder region. Rats in the control group were injected with saline. Three more booster immunizations were performed at a 2 week interval after the primary immunization. For boost immunization, 75 μg of antigen mixed in Freund's incomplete adjuvant was administered.

Blood samples were collected for anti-SRIF antibody titer through cardiac punctuation at the ages of 13 (day 15 post pregnancy) and 15 weeks (day 15 post lactation) from the five rats in each group. Sera were obtained by centrifuging fresh blood at 6,000 rpm for 20 min.

Table 1. Formula and chemical composition of experimental diet

Composition	
Formulated level	
Crude protein (%)	18.0
Digestible energy (kcal/g)	3,800.0
Ingredient (%)	
Corn, yellow	55.5
Soybean meal	27.3
Fish meal	2.0
Tallow	12.2
Vitamin-mineral mixture[1]	3.0
Total	100.0

[1] Vitamin-mineral mixture (per kg): Vitamin A, 5000 IU; Vitamin D, 1000 IU; Vitamin E, 36 mg; Vitamin K, 0.06 mg; Panthothenate-HCl, 5 mg; Thiamin-HCl, 5 mg; Riboflavin, 4 mg; Pyridoxine-HCl, 8 mg; Vitamin B12, 0.06 mg; Folacin, 1.2 mg; Cholinechlorid, 1,200 mg; $CaHPO_4$, 22.2 g; NaCl, 1.53 g; K_2SO_4, 6.70 g; MgO, 0.68 g; $FeSO_4$ $7H_2O$, 0.20 g; $CuSO_4$ $5H_2O$, 0.024 g; $MgSO_4$ H_2O, 1.21 g; $ZnCl_2$. 0.20 g.

Anti-SRIF antibody titer

Enzyme-linked immunosorbent assay (ELISA) was used to measure Anti-SRIF antibody. Each well of 96-well microtiter plates (Immulon 2; Dynatec, Laboratories Inc., Chantilly, VA) was coated with 20 mg/ml of SRIF-conjugated antigens and incubated for 18 h at 4°C. The wells were blocked with PBS containing 1% BSA for 30 min at 37°C. One hundred microliter of serum samples (1:400 dilution) were added to each well and incubated for 2 h at 37°C. Goat anti-rat-IgG peroxidase conjugated (KPL 04-16-06) was used as a secondary antibody, and TMB (3, 3', 5, 5'-tetramethylbenzidine) served as a substrate. In between the step, the wells of all plates were washed three times with PBS containing 0.05% Tween-20 (PBS-T). The absorbance of each well at 490 nm, after the addition of 0.5 M H_2SO_4 to stop the reaction, was measured using an automated spectrophotometer. Antibody levels were reported in terms of optical density (OD) readings, and expressed as means±SEM for each group.

Table 2. Peptide sequences of native 14-mer SRIF analogues

Antigen	Amino acid sequence
SRIF	Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys
Try11-SRIF	Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Tyr-Thr-Ser-Cys
Trp8Cys14-SRIF	Ala-Gly-Cys-Lys-Asn-Phe-Phe-D-Trp-Lys-Thr-Phe-Thr-Ser-D-Cys

Hormone assay

Concentration of GH in serum was measured through radioimmunoassay using BIOTRAK^{TM} (Amersham Life Science, Buckinghamshire, England).

Mammary acinar cell culture

Mammary tissue was obtained from pregnant (day 15 post pregnancy) and lactating rats (day 15 post lactation) after anesthetizing them with ethyl ether and acepromaizine maleate (PromAce, NY, USA). Mammary alveolar cells were isolated and cultured according to Frenyo (1981) with slight modification. Large pieces of connective and adipose tissues were removed, and mammary cells were placed in balanced salt solution (BSS) on ice. Mammary tissues, minced into 1 mm^3 pieces, were added into the enzyme solution (100 ml) containing 400 U/ml collagenase (Type I), 400 U/ml hyaluronidase (Type I), and 5% (vol/vol) FBS in Dulbecco's Modified Eagle's Medium (DMEM). The mixture was stirred gently at 37°C for 90 min. Digested tissue was filtered sequentially through 100- and 80-mesh sieves (Sigma, S3895 and S3770, respectively) to achieve mammary alveolar cell suspensions. The suspensions (equivalent to 3.3×10^6) were placed on 30-mm plastic culture dishes with DMEM containing 0.2% (wt/vol) glucose, 5% (vol/vol) FBS, and antibiotics (Penicillin 100 IU, Amphotericin-B 2.5 μg, Streptomycin 100 IU/ml media) (pH 7.4).

To measure the amount of milk protein synthesized, mammary alveolar cells were incubated in a basic culture medium with [^{3}H] lysine (0.5 μCi/ml) in 95% air-5% CO_2 at 37°C. After 18 h of incubation, the cells were separated via a centrifugation at 2,000×g for 10 min. Proteins in the medium were precipitated by adding trichloroacetic acid. The activity was measured from the cells or precipitated protein originated from the supernatant using a liquid scintillation counter (LS 100C, Beckman) and was expressed as dpm/mg protein. Protein concentration was measured using Lowry's method (Lowry et al., 1951).

Statistical analysis

Data were analyzed using the General Linear Model (GLM) procedure of SAS (statistical analysis system) package (SAS, 1991). Differences among the groups were evaluated through Duncan's multiple test.

RESULTS

Anti-SRIF antibody titer

Figure 1 summarizes the levels of anti-SRIF antibodies in sera of the pregnant and the lactating rats. Native SRIF and its two analogues were able to induce antibodies in the pregnant and the lactating rats. Sera from the native SRIF and Tyr11-SRIF groups showed significantly higher levels of antibodies than those from the control group, an indication that the injected with native 14-mer SRIF or Tyr11-SRIF was able to act as an immunogenic antigen in rats. The concentration of anti-SRIF antibody in SRIF and TYR11-SRIF groups was maintained significantly ($p<0.05$) higher at day 15 post-pregnancy and -lactation (figure 1). However, no difference was observed in the anti-SRIF titers of the Trp8Cys14-SRIF and the control groups, which indicates that Trp8Cys14-SRIF is not immunogenic.

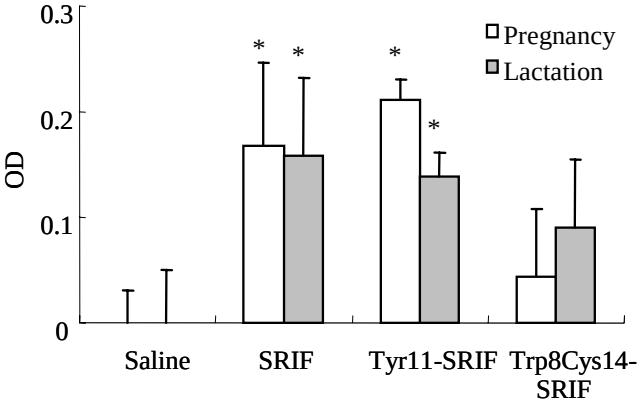

Figure 1. Level of anti-SRIF antibodies in rats immunized against saline (control), SRIF or its analogues. The values represent means±SD of quadruplicated results (n=5). Differences among the groups were tested with the Duncan's multifold range test. Asterisk (*) indicates significantly different compared to the control group at $p<0.05$.

Growth performance

To examine if the immunization against native 14-mer SRIF and 14-mer SRIF analogues influences the growth performance, total weight gain, daily feed intake, daily weight gain, and feed efficiency of the rats were measured from five to ten weeks of age. No significant differences were observed in all groups (table 3).

GH concentrations

GH concentrations in animals treated with SRIF, SRIF analogues or saline were determined from sera. No differences in serum GH concentration were observed among the treatment groups including the control group (figure 2).

Milk protein synthesis *in vitro*

To determine the effect of immunization against native 14-mer SRIF, Try11-SRIF or Trp8Cys14-SRIF on the milk protein synthesis, mammary alveolar cells obtained from the pregnant and the lactating rats were cultured for 18 h in the medium containing [^{3}H] lysine. Activities (dpm/mg protein) were evaluated from the supernatant and cells for

Table 3. Effects of immunization on total weight gain, daily feed intake, daily weight gain, and feed efficiency

	Groups				
	PBS	SRIF	TyR11-SRIF	TrP8Cys 14-SRIF	
Total weight gain (g)	88.80	97.00	90.70	92.50	NS
Day 0-14					
Daily feed intake (g)	26.11	25.97	26.01	26.39	NS
Daily weight gain (g)	3.10	3.35	3.36	3.11	NS
NS Feed efficiency	0.119	0.132	0.129	0.118	NS
Day 15-28					
Daily feed intake (g)	19.30	20.20	18.46	18.46	NS
Daily weight gain (g)	2.14	2.28	2.16	2.68	NS
Feed efficiency	0.111	0.113	0.117	0.145	NS
Day 29-35					
Daily feed intake (g)	21.17	20.44	18.64	19.02	NS
Daily weight gain (g)	2.39	2.97	2.73	2.81	NS
Feed efficiency	0.115	0.145	0.132	0.148	NS

The values are means, n=10; NS, not significant.

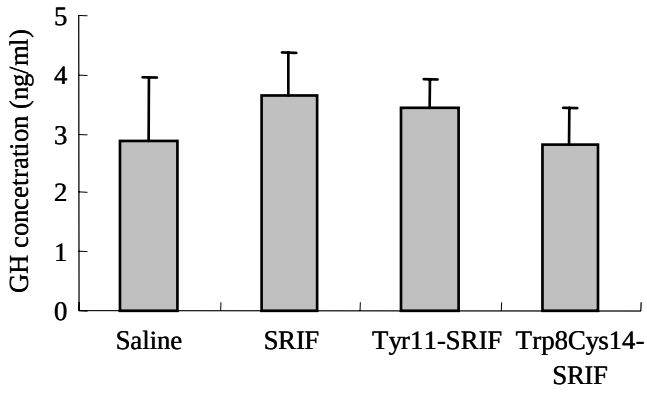

Figure 2. Effect of immunization against SRIF on the concentration of GH in rats. The values are means±SD (n=5), No significant differences were observed among the groups.

secreted and retained proteins, respectively. As shown in table 4, the activities of the test groups were not significantly different from those of the control during the pregnancy and the lactation periods. Which indicates that milk protein synthesis was not affected by immunization.

DISCUSSION

Effects of active immunization against SRIF were studied using three different forms of 14-mer SRIF. Anti-SRIF antibodies were produced from rats injected with native 14-mer SRIF or Tyr11-SRIF, but not with Trp8Cys14-SRIF. Native 14-mer SRIF and Tyr11-SRIF have been shown to induce similar pattern and amount of antibody. The level of anti-SRIF antibody production was also similar to that of 14-mer SRIF used in the previous study (Yi et al., 1999). They demonstrated that 14-mer

Table 4. Effects of immunization on the amount of secreted and retained milk proteins in the culture of mammary alveolar cells

Groups	Specific activity			
	Pregnancy		Lactation	
	Secreted protein	Retained protein	Secreted protein	Retained protein
PBS	36	73	73	138
SRIF	38	92	73	151
Tyr11-SRIF	38	73	69	140
Trp8Cys11-SRIF	48	96	87	130

* Specific activity, dpm/mg protein × 10^{-2}; The values are means, n=5.

SRIFs, regardless of forms, have no effect on the level of antibody production. However, the present study did not reveal the cause for the major difference in antibody production between native 14-mer SRIF/Tyr11-SRIF and Trp8Cys14-SRIF. It is probable that lack of antibody formation is due to differences in antigenicity and immunogenicity among the analogues. Another strong possibility is that because we used a native 14-mer SRIF as an antigen in measuring the antibody titers, Trp8Cys14-SRIF may have different structural formation compared to the native 14-mer SRIF, and therefore, Trp8Cys14-SRIF-BSA may have produced quite different group of antibodies. No differences in the anti-SRIF antibody concentrations were observed in the immunized rats during the pregnant to the lactating period. Farmer and Brazeau (1992) also revealed similar pattern using gestating gilts. They suggested that the anti-SRIF antibodies were immunoglobulin G (IgG), the main immunoglobulin class in the systemic circulation, which have a mean half-life of 10-14 days (Frenyo, 1981; Petitclerc et al., 1988). Long-

term active immunization against SRIF has been considered as a potential method to stimulate GH secretion, which, in turn, improves the growth rate in domestic livestock. This indeed is theoretically sounding. However, a number of studies have shown conflicting results. One study showed that immunization against SRIF did not affect the GH concentration in lambs (Laarveld et al., 1986), which was shown to increase in another study (Spencer et al., 1983). Magnan et al. (1995) demonstrated that rams actively immunized against SRIF showed no changes in basal and pulsatile GH secretions compared to the saline-treated control. These results suggest that the success of active immunization against SRIF is probably influenced by many factors including genotype, nutrition, species and age.

To examine the effect of active immunization against SRIF on milk protein synthesis, we performed mammary cell cultures with cells isolated from day 15 post pregnant or lactating rats. Although the level of anti-SRIF antibody was maintained from the late pregnancy till the lactation period, milk protein synthesis did not increase during this period. This result agreed with several studies (Deligeorgis et al., 1988; Yi et al., 1999), which revealed growth performance and/or milk production was not affected by active immunization against SRIF. It appears that several factors including genotype, nutrition, species, and age, influenced the effect of active immunization against SRIF on the milk production. Recent study demonstrated that immunized animals contained substantial amount of circulating endogenous hormones (Shulkes et al., 1999). In this study the average concentration of SRIF bound to the antisera from the systemic circulation of SRIF-immunized animal was 6.9 nmol/l, about 1000-fold higher than the normal levels, which indicates higher level of endogenous secretion of SRIF. Compared to the control animals, two to fourfold increase in the SRIF mRNA was in the immunized animal. Therefore, it is probable that endogenous GH secretion, upon immunization against SRIF, could be variable-dependent on the antigen preparation, adjuvant, dosage, species, age, and route of delivery.

In conclusion, active immunization against native 14-mer SRIF and Tyr11-SRIF induced SRIF antibody in the systemic circulation. However, immunization against SRIF did not affect milk protein synthesis in rat mammary gland cells.

ACKNOWLEDGMENTS

This study was supported by the NONDIRECTED RESEARCH FUND, Korea Research Foundation.

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