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Somatotropin has no Effect on the Quantity of Guanine Nucleotide Binding Proteins $G_q\alpha/G_{11}\alpha$ in Goat Adipose Tissue *in vivo*

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Summary

The decapeptide QLNLKEYNLV corresponding to the C-terminus of $G_q/G_{11}\alpha$ guanine nucleotide-binding proteins (G-proteins) was synthesized by the solid-phase method and conjugated to keyhole limpet hemocyanin. The rabbits were immunized with these conjugates and an antiserum that reacted specifically with the α subunit of G_q/G_{11} proteins was used in this study. The antiserum exhibited no cross-reactivity with the α subunits of stimulatory (G_s) or inhibitory (G_i) G-proteins associated with adenylate cyclase. Immunoblots with the antiserum showed that it specifically recognized the G_q/G_{11} α-proteins in cholate extracts of adipose tissue membranes of goats. Treatment of young castrated male goats with bST had no effect on the quantity of G_q/G_{11} α subunits in adipose tissue and the results thus obtained did not support the idea that the bST signal in adipose tissue is transmitted via G_q/G_{11} α-proteins.

Key words
Adipose tissue • Goat • Somatotropin • G-proteins

Introduction

Somatotropin (ST) is a single chain polypeptide which is produced by the somatotropic cells of the anterior pituitary gland. It is well established that ST is a homeorhetic regulator because it causes a series of coordinated metabolic adaptations, whereby a greater portion of absorbed and depot nutrients are partitioned for growth, lactation, reproduction and other functions (Bauman and Vernon 1993). The mechanisms involved have not been clearly defined. ST can act directly in tissues on the cells themselves or indirectly through the actions of locally and systemically produced IGF-I and IGFBPs (Scott *et al.* 1985, Kelley *et al.* 1996), or by

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altering the ability of cells to respond to homeostatic signals such as insulin and catecholamines (Bauman and Vernon 1993, Škarda 1998, 1999a,b). ST antagonizes insulin stimulation of glucose uptake and lipogenesis by reducing the lipogenic enzyme mass and number of glucose transporters (Bauman and Vernon 1993, Goodman 1993, Škarda 1998, 1999a,b). ST treatment *in vivo* also enhanced the release of free fatty acids from adipose tissue in response to epinephrine challenge (Sechen *et al.* 1990). Both direct and indirect actions of ST result from the initial interaction of ST with its plasma membrane receptor. However, signal transduction pathways involved in transmitting the ST signal and mediating different metabolic actions of ST are still unknown. Heterotrimeric GTP-binding proteins, comprising the α, β, and γ subunits, functionally couple hormone receptors to their signaling pathways within the cell. In ruminants, the different responses of ST-treated animals to epinephrine were not due to differences in the abundance of stimulatory ($G_s\alpha$) and inhibitory ($G_i\alpha$) subunits of G-proteins which couple β-adrenergic and adenosine receptors to adenylate cyclase. However, the functionality of G_i proteins was significantly reduced in ST-treated animals (Houseknecht and Bauman 1997). Phospholipase C (PLC) is an effector enzyme that is coupled to the receptor by other classes of G-proteins. Recent reports have identified the classes of $G_q\alpha$ and $G_{11}\alpha$ proteins as the regulators of the PLC pathway (Gordeladze *et al.* 1994).

The purpose of the present experiments was to study the effect of bovine somatotropin (bST) treatment on the quantity of $G_q\alpha/G_{11}\alpha$-proteins in omental adipose tissue of young castrated male goats.

Material and Methods

Animals and general procedures

The experiments were carried out on young castrated male goats (Czech white breed). Animals (n=12; twin brothers) were housed in a free stall barn with their dams, castrated at the age of 2 to 32 days and arranged in two groups. One of twin brothers was sometribove-treated and the second placebo-treated. Sometribove is the nonproprietary name for recombinantly derived bST consisting of the 190 amino acid form of pituitary bST with additional methionine at the aminoterminus (methionyl bovine somatotropin). Sometribove (500 mg; 22.86 μmol) was supplied as the formulated product (Somatech®) in syringes in a prolonged release (lipid-based) vehicle by Monsanto Agricultural Co. (St. Louis, MO, U.S.A.). Seven to 41 days after castration, the animals received a subcutaneous injection of placebo-olive oil (control) or sometribove (100 mg) in a sustained release vehicle at 7-day intervals for 7 to 35 days. After this treatment, all animals were exsanguinated following stunning with a captive bolt. The greater omentum was removed and immediately frozen on dry ice and kept at –30 °C until analyzed for expression of GTP-binding protein subunits.

All experimental procedures were conducted in compliance with the highest standards of humane animal care and approved by the Ethical Committee of the Institute of Animal Physiology and Genetics of the Academy.

Preparation of adipose cell plasma membranes

Tissue fragments (4 g) were homogenized using ultra-turax homogenizer (Janke-Kunkel, IKA-Werk, Staufen, Germany) in 6 ml Tris-HCl buffer (50 mmol.l^{-1} Tris-HCl; 2 mmol.l^{-1} dithiothreitol, DTT; 1 mmol.l^{-1}EDTA; 0.5 mmol.l^{-1} benzamidine.HCl; 0.5 mmol.l^{-1} phenylmethylsulfonylfluoride, PMSF; pH 7.4) and the homogenates were centrifuged at 1000x*g* for 10 min. The supernatants from this process were then centrifuged at 100 000x*g* for 60 min. The pellets were resuspended in 600 μl buffer (20 mmol.l^{-1} Tris-HCl; 25 mmol.l^{-1} NaCl; 2 mmol.l^{-1} DTT; 1 mmol.l^{-1} EDTA; 0.5 mmol.l^{-1} benzamidine.HCl; 0.5 mmol.l^{-1} PMSF) with 60 μl of 10 % sodium cholate, incubated for 2 h at 4 °C and centrifuged at 100 000x*g* for 60 min. The supernatant was adjusted at a concentration of 7-17 mg membrane protein per ml and stored at –25 °C.

Source of chemicals

Sometribove was a gift from Monsanto Agricultural Co. (St. Louis, MO, U.S.A.). Prestained SDS-PAGE molecular weight standard mixture (low range) was bought from BioRad Laboratories (Hercules, CA, U.S.A.). SDS-7B, Trizma base, dithiothreitol, benzamidine.HCl, keyhole limpet hemocyanin, Tween 20, o-phenylendiamine and glutaraldehyde were purchased from Sigma-Aldrich s.r.o. (Prague, Czech Republic). Purified holomeric G_q/G_{11}-proteins from bovine liver was a gift of Dr. J. Exton (Vanderbilt University, Nashville, TN, U.S.A.).

Boc-Lys (2-chloro-Z)-OH, Boc-Glu(Octtex)-OH, Boc-Tyr(Z-bromo-Z)-OH, Boc-Glu OH, Boc-Leu OH.H_2O, Boc-Asu OH, Boc-Val OH and Merrifield Polymer chross-linked with 1 % divinylbenzene resin (100-200 mesh) were bought from BACHEM (Buchendorf, Switzerland). Divinylbenzene, dichloromethane, dicyclohexylamine,

2-diisopropylaminoethyl-amine, hydrofluoric acid, dimethylaminopyridine, trifluoroacetic acid and dimethylformamide were purchased from Fluka Chemie AG (Buchs, Switzerland). Bovine serum albumin was obtained from SEVAC (Prague, Czech Republic).

Peptide synthesis

C-terminal decapeptides of G_q subunits of known amino acid sequences for G_q/G_{11}-QLNLKEYNLV, G_s-RMHLRQYELL and $G_{i1,2}$-KENLKDCGLF were assembled stepwise by the solid-phase method (Barany and Merrifield 1979). Peptide resins were cleaved with anhydrous hydrogen fluoride and anisole (9:1) at 0 °C, and the crude peptides were purified on reverse-phase columns (250 x 4 mm) of Sepharone SG x C18 (10 μm) (Knauer, Berlin, Germany) on analytical HPLC (Spectra Physics, Fremont, CA, U.S.A.). The purified peptides were homogeneous on analytical chromatogram and gave amino acid compositions consistent with those theoretically expected. Amino acid sequences were verified by amino acid analysis and corresponded to those theoretically expected. Peptides gave a single band on thin layer chromatography (Silufol, Kavalier, Sázava, Czech Republic) in the solvent system n-butanol, acetic acid and water (4:1:1, v/v).

Peptide conjugation and immunization

Peptide conjugation was performed by coupling to keyhole limpet hemocyanin (Goldsmith *et al.* 1987).

Conjugates were emulsified with an equal volume of complete Freund's adjuvant and the resulting fresh emulsion (1 mg of peptide) was injected intramuscularly in three stabs into rabbit gluteal muscles, and this was repeated twice with the same antigen emulsified in incomplete Freund's adjuvant at intervals of 21 days. Eight days after the last injection animals were exsanguinated. The titre of antibodies was determined by ELISA. Antisera were tested for reactivity with the purified holomeric G_q/G_{11}-proteins from bovine liver following resolution in 12 % (w/v) polyacrylamide SDS-PAGE on immunoblots.

Immunoblotting (Western blotting)

Membranes were resolved by SDS-PAGE in 14 x 16 cm slab gels using the 13 % (w/v) acrylamide, 0.25 % bisacrylamide system at 45 V for 12 h at 4 °C with subsequent electrotransfer to PVDF transfer membrane (BioRad Lab.) using a semidry transfer system CARBOGLASS (Schleicher & Schuell) at 300 mA for 3 h. After blocking for 2 h with 3 % BSA at room temperature in PBS (0.01 $mol.l^{-1}$ phosphate buffer, 0.0027 $mol.l^{-1}$ potassium chloride and 0.137 $mol.l^{-1}$ sodium chloride, pH 7.4 at 25 °C) followed washing with PBS containing 0.2 % Tween 20 (1 x 1 min; 1 x 15 min; 2 x 5 min). Then the first antibody diluted 1:2500 in PBS containing 1 % of BSA was added and incubated for 1 h at room temperature. The first antibody was then removed and this was followed by washing with PBS containing 0.2 % Tween 20 (1 x 1 min; 1 x 15 min; 2 x 5 min) and incubation with second antibody (swine antibody IgG) conjugated with horseradish peroxidase and diluted 1:5000 in PBS containing 1 % BSA (3 h at room temperature). After washing with PBS containing 0.2 % Tween 20 (1 x 1 min; 1 x 15 min; 2 x 5 min) immunoblots were stained with 3,3'o-diaminobenzidine in a citrate buffer (pH 5.5) with H_2O_2 for 15 min at room temperature. Staining was stopped by the addition of 1 % NaN_3 in bidistilled water. The developed immunoblots were scanned with a BioRad GS-700 imaging densitometer (BioRad Lab.) in the reflectance mode (400-750 nm). The results were analyzed using BioRad Lab. Molecular Analyst Program enabling quantitation of the immunoblots and analysis of the relative expression of G_q/G_{11}-proteins in bST-treated and control animals. The results were expressed as mean arbitrary optical density units taken from densitometric scanning of Western blots.

Protein determination

The protein content of adipose tissue membranes was determined according to Lowry *et al.* (1951).

Statistical methods

The optical densities of G_q/G_{11}-proteins of adipose tissue of control and bST-treated animals are expressed as mean values ± S.E.M. Comparisons were performed using the non-paired t-test.

Results

We synthesized decapeptides, QLNLKEYNLV, RMHLRQYELL and KENLKDCGLF, corresponding to the carboxylterminus of $G_q\alpha/G_{11}\alpha$-, $G_s\alpha$-, and $G_{i1,2}\alpha$-proteins, respectively. Peptides were then conjugated either to keyhole limpet hemocyanin. Antipeptide polyclonal antisera were raised in rabbits. Cross reactivity of antisera G_q/G_{11} with antisera against G_s- and $G_{i1,2}$-protein was assayed by ELISA. No cross reactivity was found as extinctions at 492 nm were between 0.04 and 0.09. G_q/G_{11} antiserum was tested for specific reactivity with a mixture of purified holomeric G_q/G_{11}-proteins from bovine liver in parallel with membranes from goat adipose tissue (Fig. 1). The age of animals and the

duration of bST treatment did not affect the magnitude of the response of G_q/G_{11}-proteins. Hence, the amounts of G-proteins obtained were pooled separately for control and bST-treated animals. The treatment with bST had no significant effect on the quantity of G_q/G_{11} α-proteins (Fig. 2, Table 1) in omental adipose tissue membranes.

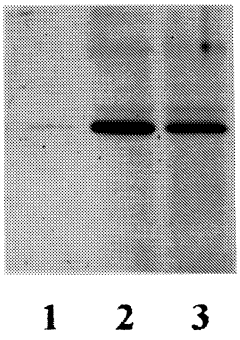

Fig. 1. *Antiserum generated against a synthetic C-terminal decapeptide which is common to the α subunits of G_q and G_{11} identifies a mixture of purified holomeric G_q/G_{11} proteins from bovine liver and goat adipose tissue, Lane 1: a purified mixture of holomeric G_q/G_{11} proteins from bovine liver (the kind gift of Dr. J. Exton). Lanes 2 and 3 represent G_q/G_{11} proteins from membranes of goat adipose tissue: lane 2 – bST-treated animal, lane 3 – placebo-treated animal, 100 μg protein per lane.*

Table 1. Effect of bovine somatotropin (bST) treatment on the amount of G_q/G_{11} α-proteins in goat adipose tissue.

	G_q/G_{11} α-proteins (in arbitrary optical density units)		**Effect of bST treatment %**
	Control	**bST-treated**	
	5.55	4.64	–16
	7.36	7.01	–5
	7.24	9.94	37
	5.39	7.42	38
	4.81	6.70	39
	49	4.14	66
Mean	5.47	6.64	27
±S.E.M	±0.73	±0.85	

Six pairs of twin brothers were castrated at age of 2 to 32 days and arranged in two groups for the treatment (placebo-treated and bST-treated). Starting 7 to 41 days after castration, one animal from each pair received an s.c. injection of olive oil (control) and one with recombinant bST in a lipid-based sustained release vehicle (100 mg) at 7-day intervals for 7 to 35 days. After treatment, the greater omentum was removed and analyzed for G-protein expression.

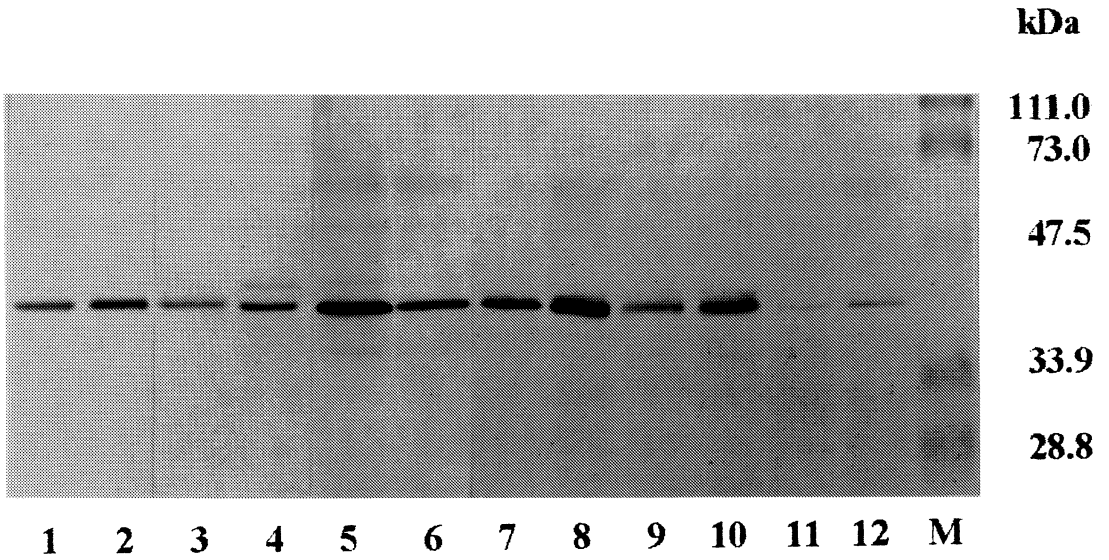

Fig. 2. *Detection of G_q/G_{11}-proteins in omental adipose tissue of placebo- and bovine somatotropin (bST)-treated young castrated male goats. Six pairs of twin brothers were castrated at the age of 2 to 32 days and arranged in two member blocks for treatment assignment (placebo-treated and bST treated). Starting 7 to 41 days after castration one animal was subcutaneously injected with placebo (olive oil) and one animal was injected with 100 mg of recombinant methionyl bST in a lipid-based sustained release vehicle (day 1 of treatment) at 7-day intervals for 7 to 35 days. Animals were killed on day 10 of treatment. Immunoblots were performed as described under Materials and Methods. The position of molecular weight standard is indicated (M) from the top 111.0, 73.0, 47.5, 33.9 and 28.8 kDa of molecular weights, respectively. In lanes 1, 3, 5, 8, 10 and 12 were loaded membrane proteins from bST-treated animals; in lanes 2, 4, 6, 7, 9 and 11 were loaded membrane proteins from control animals. Lanes 1-10: 100 μg protein; lanes 11 and 12: 50 μg protein.*

Discussion

ST acts to decrease the adiposity in ruminants by several mechanisms including a diminution of lipogenesis (a decrease of acetyl CoA carboxylase and of fatty acid synthase), antagonizing insulin stimulation of lipogenesis, enhancing β-adrenergic inhibition of lipogenesis and that of lipolysis (Sechen *et al.* 1990, Borland *et al.* 1994, Wilson *et al.* 1996, Etherton and Bauman 1998, Škarda 1998, 1999a,b). It was found in ovine adipocytes that ST did not activate MAPK and PI3 kinase so that these pathways are not apparently involved in the homeorhetic effects of ST (Vernon 1997) and that the chronic homeorhetic effects of ST are thus mediated by intracellular signaling systems distinct from those mediating other ST effects in adipocytes and preadipocytes (e.g. insulin-like effect of ST, MAPK).

To elucidate further the molecular basis of the ST action in ruminant adipose tissue, Doris *et al.* (1996) and Houseknecht and Bauman (1997) studied the abundance of G-proteins in ST-treated sheep and cows. The ST treatment did not alter the abundance of both $G_s\alpha$- and $G_i\alpha$-proteins. This is in agreement with the results of our study on goat adipose tissue (unpublished observations). However, the functionality of the G_i-proteins, as assessed by their ability to be ADP-ribosylated by the pertussis toxin, was significantly reduced with ST treatment (Doris *et al.* 1996, Houseknecht and Bauman 1997). Thus, the enhanced lipolytic response to catecholamines observed *in vivo* in ST-treated animals is, to a large extent, related to the relief of tonic inhibition of lipolysis *via* changes in the G_i signaling cascade. Whether this alteration in G_i-protein function also leads to a decrease in lipogenesis remains to be established.

The major objective of this study was to determine the effect of bST treatment *in vivo* on the quantity of PLC-coupled $G_q\alpha/G_{11}$ α-proteins in goat adipose tissue. The involvement of PLC and protein kinase C in ST inhibition of lipogenesis in sheep adipose tissue have already been demonstrated (Vernon 1996). However, bST treatment *in vivo* had no effect on the quantity of G_q/G_{11} α-proteins in omental adipose tissue of young goats (present results). Preliminary results obtained in *in vitro* cultured goat omental adipose tissue have also not shown any stimulatory effect of bST on the quantity of G_q/G_{11} α-proteins (our unpublished results).

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