Tumor Necrosis Factor-α Affects Growth Hormone Secretion by a Direct Pituitary Interaction (43287)

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Abstract. **Administration of 50, 250, and 1,250 ng/kg iv of recombinant bovine tumor necrosis factor-α (RBTNF) did not affect basal plasma concentrations of growth hormone (GH) or thyroid-stimulating hormone in male calves. However, when administered 30 min before challenge with 1 μg/kg iv of thyrotropin-releasing hormone (TRH), 250 ng/kg of RBTNF increased the subsequent incremental GH response. At 1,250 ng/kg of RBTNF, GH response to TRH was significantly blunted. For each dose of RBTNF administered, the incremental change in plasma thyroid-stimulating hormone following TRH was not significantly different from control. To examine direct effects of RBTNF on pituitary function, fresh bovine pituitaries were sliced into 1-mm cubes and incubated with 0 or 10^{-8}, 10^{-9}, or 10^{-10} *M* RBTNF. Additional cultures were treated with 10^{-8} or 10^{-9} *M* GH-releasing factor or 10^{-8} *M* TRH and 0 or 10^{-8} *M* RBTNF. Media GH increased in cultures with 10^{-10} *M* RBTNF and declined linearly as RBTNF concentration increased. RBTNF blocked GH release from GH-releasing factor- and TRH-challenged pituitary slices. Membranes prepared from homogenized bovine pituitaries had specific saturable binding characteristics for monomeric ^{125}I-RBTNF. Membranes treated with 4 *M* $MgCl_2$ for 10 min and washed free of Mg^{2+} produced Scatchard plots fit to a two-site model (high affinity site K_d = 6.6 n*M*), while Scatchards of non-Mg^{2+}-treated membranes fit a single site (K_d = 8.9 n*M*). Polyacrylamide gel electrophoresis separation of ^{125}I-RBTNF cross-linked pituitary membranes showed specific binding of monomeric ^{125}I-RBTNF to protein components ranging in molecular weight from 19,000 to 77,000. The data suggest that RBTNF has modulatory effects on the regulation of GH secretion acting directly at the pituitary through specific receptors.** [P.S.E.B.M. 1991, Vol 198]

Much attention is presently being focused on the role of cytokines as communication vectors between the immune and endocrine systems (1-4). Attempts to model the effects of interleukins (IL) and tumor necrosis factor-α (TNF) interacting with hormone systems have ranged from administration of endotoxins and cytokines *in vivo* to direct application on cultured cells and tissues (5-11). However, there are few data that address the mechanism by which cytokines affect pituitary hormone secretion. While changes in plasma hormones in association with administration of IL have been demonstrated, results have been inconsistent (6, 7). Data on the *in vivo* effects of TNF on plasma hormones are, at this time, very limited in any species. Previous data from our laboratory demonstrated that administration of 5 mg/kg iv of recombinant bovine TNF (RBTNF) to calves resulted in a rapid decline in circulating growth hormone (GH) and luteinizing hormone (11) similar to that observed when calves were challenged with 055:B5 coliform endotoxin, which released endogenous TNF. However, even when purified cytokines are singly administered *in vivo*, interpretation of data can be somewhat confounded. For example, TNF has been shown to elicit IL-1 release from macrophages (12), which, in itself, could affect pituitary secretion. Because IL-1 and TNF affect carbohydrate and fat metabolism, effects on pituitary hormone secretion secondary to acute metabolic disturbance cannot be ruled out. Particularly in the case of GH secretion, insulinemia, hypoglycemia, hyperglycemia, and hyperlipidemia affect GH responses to natural secretagogues (13, 14). The present study was therefore undertaken to examine possible mechanisms through which TNF

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affects pituitary function and to determine whether TNF interacts with known pituitary secretagogues to modify the hormone-releasing character of these peptides.

Materials and Methods

***In Vivo* Challenge.** Three to five-month-old Holstein bull calves were used to assess the effect of intravenous administration of RBTNF on basal- and thyrotropin-releasing hormone (TRH)-stimulated plasma GH and thyroid-stimulating hormone (TSH) concentrations. Calves were fed a diet that consisted of hay and a corn-soy bean meal concentrate mixture to effect a low to moderate growth rate but that always maintained a positive nitrogen balance. At this plane of nutrition, pulsatile GH secretion (pulse frequency, peak amplitude of secretory spikes, and overall mean concentration) is suppressed relative to that measured in calves fed to a significantly higher plane of nutrition (15). The attenuation of the random (in bovine) pulsatile GH secretion is important for quantifying the GH response to secretagogue stimulation, because the magnitude of the response is affected by when TRH is administered relative to the peak and trough of the secretory episode (16, 17). All blood samples were obtained from, and administrations were made into, the jugular vein by venipuncture while calves were gently hand restrained. The number of bull calves used for each dosage of RBTNF varied between four and six, depending on the temperament of the animals on the day of sampling.

Previous experiments in our laboratory demonstrated that significant changes in plasma GH and luteinizing hormone were apparent within 30 min of administration of RBTNF (5 mg/kg) to calves (11). Additional data demonstrated that the GH peak response to TRH in calves of this age occurred 10 to 15 min after TRH administration (18). Because of variability in basal GH and GH responses to TRH challenge, the dose of TRH (1 μg/kg), plane of nutrition, and timing of blood sampling relative to feeding were chosen to increase the uniformity of incremental hormone responses to TRH between calves. Recombinant bovine TNF-α was produced by Genetech (San Francisco, CA) and obtained by a contractual arrangement through Ciba-Geigy (St. Aubin, Switzerland). Thyrotropin-releasing hormone was obtained from Sigma Chemical Co., (St. Louis, MO). A blood sampling-dosage regimen was established in which three preliminary blood samples (T-20, T-10, and T-00) were collected at 10-min intervals prior to administration of RBTNF or saline control vehicle. Additional blood samples were obtained from calves 30 min after T-00 (T + 30), at which time TRH (1.0 μg/kg iv) was administered. Final blood samples were obtained at 10 and 20 min (T + 40 and T + 50) after TRH. All treatments were administered in a volume of 2.0 ml. RBTNF was diluted from a stock solution with sterile 0.9% saline. Increasing dosages of RBTNF (0, 50, 250, 1250 ng/kg body wt) were administered consecutively on a weekly basis. The use of these doses, in contrast to the 5 mg/kg dose used previously (11), was based on the need to focus on the role of TNF in pituitary function. It was necessary to separate possible physiological functions from effects that were confounded with pulmonary and circulatory complications of shock (similar to endotoxic shock) commonly experienced with TNF administration at higher doses. A master stock of TRH was prepared (diluted in 0.9% saline containing 0.1% bovine serum albumin), aliquoted, and frozen. For each day's TRH challenge, TRH was freshly thawed. Blood sampling was initiated 30 min after morning feeding. Plasma was obtained from blood collected into EDTA and stored frozen until assayed for GH and TSH by specific radioimmunoassays (15, 18). Changes in basal plasma GH and TSH concentrations following RBTNF were compared with control saline-treated calves (mean of T-20, T-10, T-99 vs T + 30). Similarly, the increments (peak at T + 40 or T + 50 minus mean basal) in plasma GH and TSH following TRH were compared among doses of RBTNF using analysis of variance (General Linear Models Procedure of SAS and specific orthogonal contrast [19]), statistically tested with dose of RBTNF as a main effect. Data are presented as means with relative variance presented as the standard error of the mean. Results were significant if $P < 0.05$, with significant trends suggested when $P < 0.1$.

***In vitro* Challenge.** Fresh bovine anterior pituitaries were obtained from four Holstein cows, transported to the lab in Dulbecco's modified Eagle's medium, and sliced into cubes (approximately 1 mm on side). Pituitary slices from all cows were mixed together so that selection of slices for each well was random with respect to animal. Three pituitary slices were incubated in each chamber of 24-well plastic culture dishes containing 1.0 ml of Dulbecco's modified Eagle's medium. Slices were incubated and equilibrated at 37°C in 5% CO_2, and washed twice with fresh Dulbecco's modified Eagle's medium. The final 1.0-ml volume contained the same medium supplemented with the various treatment hormones as outlined: (i) control media; (ii) 10^{-9}, or 10^{-8} *M* growth hormone-releasing hormone (GRF; peptide fragment 1–40; Peninsula Lab, Blemont, CA) or TRH (10^{-8} *M*); (iii) 10^{-10}, 10^{-9}, or 10^{-10} *M* RBTNF; or (iv) 10^{-8} *M* GRF or TRH plus 10^{-8} *M* RBTNF (N = 3–4 wells/treatment). Treatments were applied for 3 hours. Media were collected and centrifuged and the supernatant was frozen and subsequently assayed for GH by radioimmunoassay. The effects of GRF, TRH, and dose of RBTNF on media GH concentrations were statistically compared by analysis of variance, with treatment and dose as main effects in the model.

Receptor Assay. Fresh bovine pituitaries were obtained from Holstein cows, minced into pieces, and dispersed in 9 vol of 250 mM sucrose containing aprotinin (1,000 KIU/ml). A homogenate was prepared using a Polytron at Setting 5 for 10 sec and Setting 10 for 20 sec, and centrifuged at 12,000g for 20 min. The supernatant was removed and the ionic strength was increased with the addition of solid NaCl and $MgCl_2$ to obtain 0.1 M NaCl and 0.005 M $MgCl_2$, respectively. This fraction was centrifuged at 105,000g for 40 min and the resultant supernatant was discarded. The pellet (membrane fraction) was redispersed in buffer containing 0.01 M phosphate, 0.1 M sucrose, 20 mg/liter of merthiolate, and 0.1% Tween 20 (pH 7.4; binding buffer), and divided into equal portions. To one portion, solid $MgCl_2$ was added to a final concentration of 4 M. Membranes were exposed to the Mg^{2+} for 10 min, after which the preparation was centrifuged at 14,000g for 10 min and the pellet washed in binding buffer. The other membrane portion was not subjected to Mg^{2+} treatment. The membrane protein content of the homogenate was estimated by the Bradford dye binding method (20). Membrane protein concentration was adjusted so that 90 μg of protein were added to each tube. For binding studies, binding buffer was used, but with the $[M^{2+}]$ adjusted to 0.005 M and radioimmunoassay grade bovine albumin (2% w/v) added.

Recombinant bovine TNF was iodinated (10), resulting in a sp act of 89 μci/μg, and monomeric ^{125}I-RBTNF was obtained following separation with Sephadex G-75 (20–50 M, 0.7 × 25 cm) eluted in assay buffer (10). The specific activity was calculated based on the principle of self-displacement (21) by using increasing volumes of tracer in a well-validated radioimmunoassay specific for bovine TNF (10). The specific activity arrived at by this method was in good agreement with the theoretical specific activity calculated from the mass of hormone iodinated, the quantity of radioiodine used, yield, recovery, and losses. Additional binding checks were performed using elution fractions corresponding to dimeric and trimeric RBTNF. The highest membrane binding with the lowest nonspecific binding was achieved with eluted fractions corresponding to a mol wt of 17,000 (monomeric TNF). Initial characterization of binding was accomplished by adding increasing concentrations of labeled RBTNF to membranes with and without excess (10 μg RBTNF, nonspecific binding), equilibrating binding over a 14-hr period. For characterization by Scatchard analysis, binding was evaluated by incubating membranes with 50,000 cpm of ^{125}I-RBTNF and increasing concentrations of nonlabeled RBTNF. Binding assays were performed in 1.5 ml of polypropylene tubes at 4°C for 14 hr. Membrane-bound and free ^{125}I-RBTNF were separated by centrifugation at 14,000g for 10 min. Supernatant was aspirated and the bound radioactivity in the pellet was counted. Binding characteristics were ascertained using the LIGAND program (22) with initializing estimates of $K_1 = 3 \times 10^{-10}$ and $K_2 = 5 \times 10^{-8}$ (in the two-site model), respectively, with nonspecific binding subtracted prior to analysis.

TNF Receptor Size. To evaluate relative sizes of membrane components that bind RBTNF, 90 μg of membrane protein were incubated with 500,000 cpm of ^{125}I-RBTNF characterized as monomeric by gel chromatography (equilibrium conditions) ± 10 μg nonlabeled RBTNF. Following a brief washing, tracer was covalently bound to membrane receptors using 0.2 mM final concentration disuccinimidyl suberate (DSS) for 15 min at 4°C in 50 mM phosphate buffer (pH 7.5). At the end of the incubation time, the reaction was stopped by the addition of 25 mM Tris and 1 mM EDTA (pH 7.0). Membranes were solubilized with sodium dodecyl sulfate and electrophoresed under reducing and nonreducing conditions. Gels were vacuum dried and subjected to autoradiography. Specific bands were compared with Coomassie blue-stained molecular weight standards in the same gel. Additional checks on the validity of the labeling and autoradiographic-banding patterns were obtained by slicing and counting gels for radioactivity and by probing membrane proteins electrophoresed onto nitrocellulose paper with labeled TNF and performing autoradiography.

Results

***In Vivo* Challenge.** The effects of administration of 0, 50, 250, or 1250 ng/kg of RBTNF on TRH-induced changes in plasma GH and TSH are depicted in Figure 1. The administration of saline to calves had no perceptible effect on mean circulating basal plasma concentrations of GH or TSH. When GH and TSH concentrations measured 30 min after administration of 50, 250, or 1250 ng/kg of RBTNF were compared with pre-TRH hormone concentrations, there were no significant differences. Averaged across 50, 250, and 1250 ng/kg doses of RBTNF, plasma concentrations of GH were 2.45 ± 0.21 and 3.27 ± 0.31 ng/ml, before and after administration of RBTNF; plasma TSH concentrations averaged 0.55 ± 0.04 and 0.52 ± 0.05 ng/ml before and after RBTNF, respectively.

Administration of TRH to calves resulted in significant increases in plasma GH and TSH in all calves. The increment in plasma GH following TRH increased as the dose of RBTNF increased through 250 ng/kg ($P < 0.05$). At the 1250-ng/kg dose, the GH response to TRH was significantly attenuated ($P < 0.05$) compared with the 250-ng/kg RBTNF dose. Also, at this dose of RBTNF, there was a significant trend toward reduced GH response to TRH relative to that observed at the 0-RBTNF dose ($P < 0.1$). The mean increment in plasma TSH following TRH varied between 11 and 18 ng/ml, with no uniform trend associated with the various doses

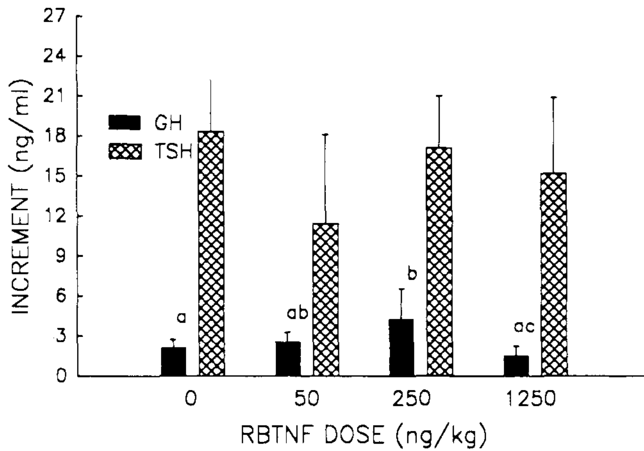

Figure 1. Mean incremental responses (± SE) in plasma GH and TSH following intravenous TRH (1 μg/kg, iv) in calves, relative to pretreatment with various doses of RBTNF (n = 4-6 calves/dose). Various doses of RBTNF (0, 50, 250, or 1250 ng/kg, iv) were administered at weekly intervals, with doses increasing over time. TRH was administered 30 min later. The effects of RBTNF on the incremental responses to TRH were analyzed by comparing the increment in control ("0" dose level) with the response at each dose level. The maximum increment was defined as the difference in peak GH or TSH concentration value following TRH minus the mean basal plasma GH or TSH values as averaged across the three pretreatment samples. Figure bars with different letters are significant (P < 0.05).

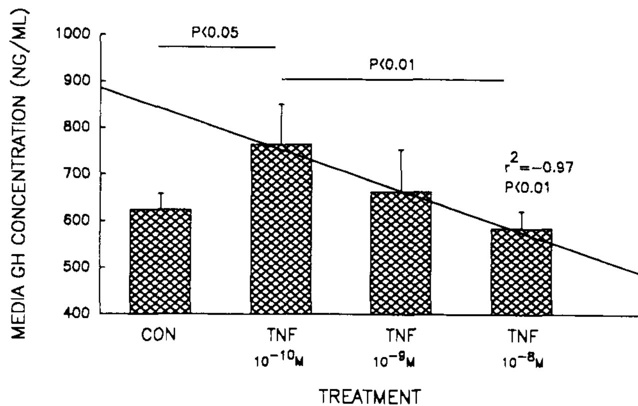

Figure 2. Effect of RBTNF (10^{-10}, 10^{-9}, and 10^{-8} M) on mean basal GH concentrations in media from *in vitro* incubated bovine anterior pituitary slices (n = 3-4 wells/treatment). Media basal concentrations of GH were significantly (P < 0.01) negatively correlated with increasing concentration of RBTNF.

of RBTNF administered. In addition, calves were not distressed by any of the RBTNF doses, as evidenced by physical appearance and a willingness to continue eating. At the highest dose of RBTNF, increased heart and respiratory rates were evidenced, but no change in rectal temperature was measured (data not presented).

***In Vitro* Challenge.** The concentration of GH in media from pituitary slices incubated with 10^{-8} M RBTNF was not statistically different from control values (Fig. 2). At 10^{-10} M, RBTNF increased media GH concentrations compared with GH from pituitary slices cultured with no RBTNF or 10^{-8} M RBTNF (P < 0.02). Increasing concentrations of RBTNF were negatively correlated with mean media GH concentration (P < 0.01, r^2 = −0.97). GRF increased media GH in slice preparations at 10^{-8} M (P < 0.02; Fig. 3). At 10^{-8} M, RBTNF abolished the GH secretagogue effect of GRF (P < 0.02). Similar to the effect of 10^{-8} M RBTNF on the GH response to GRF, RBTNF blocked the action of TRH to increase media GH concentration (P < 0.05; Fig. 3).

TNF Receptors. Plasma membrane receptors displayed specific and saturable binding of iodinated RBTNF (Fig. 4A). Absolute total binding was higher with membranes that had been treated previously with $MgCl_2$ to theoretically remove bound endogenous TNF from receptors. When membranes were pretreated with 4 M $MgCl_2$, a two-site model (Fig. 4C) fit the data better (lower residual variance) than a single-site model (Fig. 4B), yielding a high affinity site with K_d = 6.6 nM (Fig. 4C). Scatchard analysis of the binding data from non-Mg^{2+}-treated membranes (Fig. 4B) suggested a single-site best fit with an affinity of 8.9 nM. Human recombinant TNF-α displaced ^{125}I-RBTNF with a slope not different from that obtained with increasing concentrations of the RBTNF, but increasing concentrations of human TNF-β, human γ-interferon, and interleukin 2 failed to displace bound ^{125}I-RBTNF from pituitary membranes (Fig. 5).

Receptor Molecular Size. Autoradiography following polyacrylamide gel electrophoresis demonstrated specific receptor binding components with relative molecular wt of 19,000, 40,000, 58,000, and 77,000 following adjustment for the molecular weight of the bound tracer (Fig. 6). Similar bands were detected with other confirming methods, but a faint band at 130 kDa found with sodium dodecyl sulfate-polyacrylamide gel electrophoresis was not apparent by the other methods tested (data not presented).

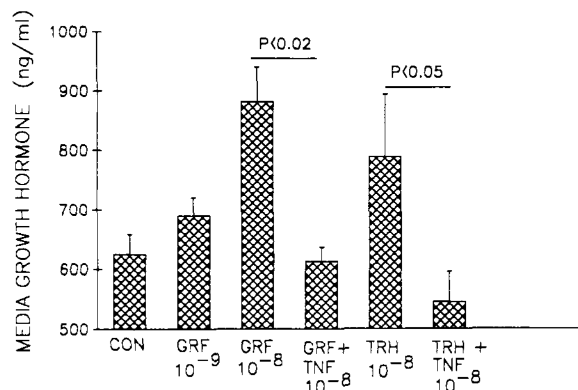

Figure 3. Effect of GRF (10^{-9} and 10^{-8} M) alone and in combination with RBTNF (10^{-8} M) on GRF (10^{-8} M)- and TRH (10^{-8} M)-stimulated media concentrations of GH from incubated bovine anterior pituitary slices.

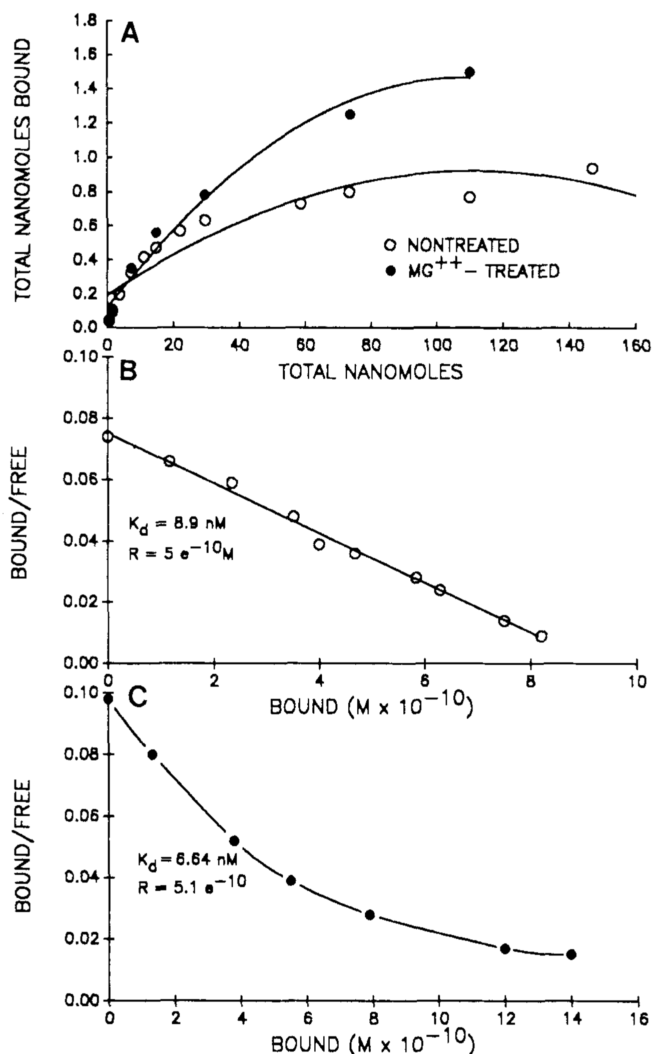

Figure 4. Binding of ^{125}I-labeled RBTNF to pituitary membrane homogenates. (A) The effect of pretreatment of membranes with 4 *M* $MgCl_2$ for 10 min prior to binding assay on total binding of RBTNF to pituitary homogenate membranes. (B) The single-site model of binding obtained when pituitary homogenate membranes were not pre-exposed to 4 *M* Mg^{2+} (K_d = 8.9 n*M*). (C) The Scatchard plot of the binding data from Mg^{2+}-treated membranes as a two-site model with a high affinity site of 6.6 n*M*.

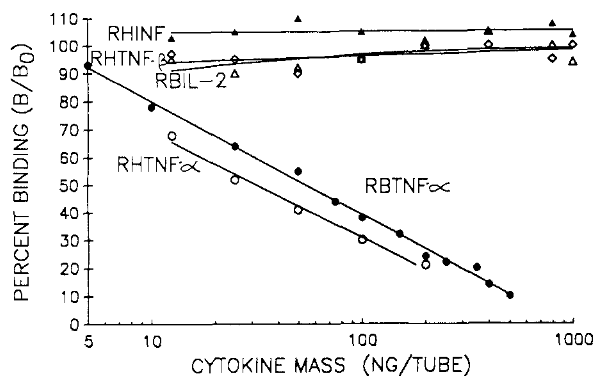

Figure 5. Relative cross-reactivity of the pituitary TNF receptor with other related cytokines represented by the ability of recombinant human TNF-α (RBTNF-α), recombinant bovine interleukin 2 (RBIL-2) and recombinant human γ-interferon (RHINF), and recombinant human TNF-β (RHTNF-β) to displace labeled bovine TNF from pituitary homogenate membranes. Similarity in displacement slopes between RBTNF and RHTNF-α suggest a similar affinity, while the specificity of the receptor for TNF-α is inferred by the lack of displacement of tracer by other cytokines.

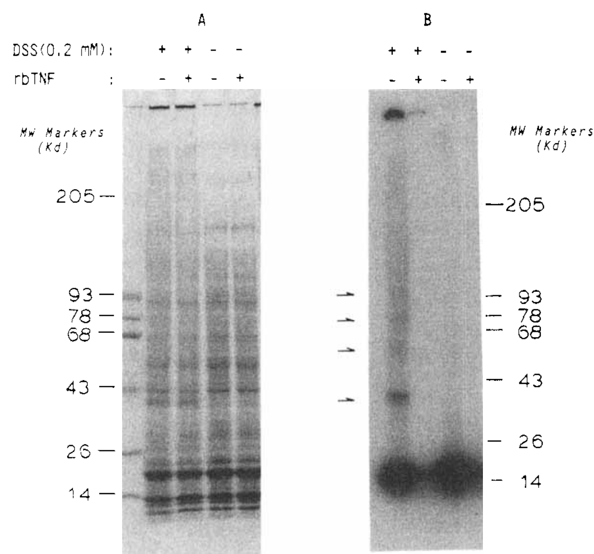

Figure 6. Banding patterns as relative indices of molecular size of anterior pituitary membrane proteins. (A) Coomassie blue-stained electrophoretic profiles (sodium dodecyl sulfate-polyacrylamide gel electrophoresis, 5–15% linear gradient) of cross-linked (0.2 m*M* disuccinimidyl suberate [DSS]) and noncross-linked bovine pituitary membranes. (B) The autoradiogram of affinity-labeled bovine pituitary membranes incubated with ^{125}I-RBTNF ± 10 mg of nonlabeled RBTNF followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Arrows represent bands that are specifically labeled with ^{125}I-RBTNF and correspond to polypeptides that are 19, 40, 58, and 77 kDa when the migration attributable to the 17-kDa RBTNF is subtracted from each.

Discussion

In our *in vivo* studies, RBTNF had no measurable effect on basal concentrations of GH or TSH, or on the incremental TSH response to TRH in calves. Particularly for GH, any measurable change in basal GH was at least absorbed in the normal variability in plasma GH associated with pulsatile secretion. However, RBTNF had an apparent biphasic effect on the magnitude of GH responses to TRH. At the lower doses tested (50 and 250 ng/kg), RBTNF increased the GH response to a standard challenge with TRH. At the highest dose tested, the GH response to TRH was significantly blunted. The incremental responses to this dose of TRH were relatively low, but similar to those measured previously in calves fed to a similar plane of nutrition (18). It would appear that the role of TNF in pituitary function is a modulatory one in nature, rather than that of a major effector such as GRF or TRH. The physiological relevance of these subtle effects of TNF on the plasma GH response to TRH are still sought. However, the incremental plasma GH response to TRH at the 0-RBTNF dose represents a 2-fold

increase over baseline concentrations, whereas the GH response to TRH was 4-fold baseline concentrations in conjunction with the 250 ng RBTNF/kg dose.

We find it interesting that the effect of TNF on secretagogue action in this study was confined primarily to the GH response. This might reflect TNF effects on GH secretion at hypothalamic, as well as pituitary, sites of regulation. This may be possible because of demonstrated effects of other cytokines on pituitary hormone secretion effected through intracerebroventricular administration (5). It is also possible that the effect of TNF on TSH release in this particular experiment may have been masked due to the strong release of TSH at the dose of TRH used. However, administration of TNF to rats has been demonstrated to impair thyroid function without significant effects on TSH release subsequent to TRH challenge (23). The magnitude of the GH responses to TRH here may have been more conducive to detecting an effect of TNF that was lost in a more overt TSH response to the TRH.

A dose of 5 mg/kg of RBTNF used in earlier studies (11) depressed plasma GH concentrations to nondetectable levels by radioimmunoassay within 1 hour of administration; but it was accompanied by significant increases in respiratory rate, heart rate, and salivation, as well as decreased feed consumption and prolonged hypoglycemia. The overt effect on GH secretion may have been due to several factors, including: direct action on the pituitary; associated effects of other cytokines released by RBTNF; acute metabolic perturbations; and possible ischemic responses associated with the acute cardiopulmonary effects characteristic of, and mimicked by, endotoxin challenge. The acute response to administration of either endotoxin or TNF (in high enough doses) is rapid in onset (2–5 min). It is characterized by shortness of breath with labored breathing, rales upon pulmonary auscultation, increased heart rate, sweating, and pulmonary hypertension during peripheral hypotension (24, 25). It was important in the present study to avoid doses of TNF that would produce these adverse responses. While the lower doses of RBTNF used in the present study did not significantly affect basal plasma GH, pretreatment of calves with RBTNF did affect the GH release subsequent to administration of TRH. The lack of an observable and measurable effect of RBTNF on basal GH and TRH plasma concentrations at the doses used suggests that the role of TNF in pituitary regulation is probably not a primary one. The observable effects on plasma GH and luteinizing hormone at 5 mg/kg reflect a severe perturbation of homeostatic mechanisms that are beyond usual physiological bounds, but may be important considerations in cases in which pathology is evident.

Often in research, responses measured *in vivo* are not exactly parallel to those obtained using an *in vitro* method. Significantly more dynamic and complex interactions *in vivo* are usually not mimicked *in vitro*. In the case of TNF effects on hormone secretion, indirect effects of metabolic shifts associated with TNF administration might interact to modify measured hormone responses. In the present study, indirect effects of RBTNF on pituitary function were basically eliminated with the use of isolated *in vitro* slice studies. Interestingly, and in contrast to that seen *in vivo*, low doses of RBTNF increased media GH concentrations. This contrasts with what was observed by Walton and Cronin (9), but the attenuation of GH release by GRF and TRH into media in the presence of RBTNF agrees with their findings. Contributing to the difference in findings between these two studies is the fact that slice cultures were used in our study, as opposed to enzymatic dispersed cells; also, the species origin of the tissue differed. Other data from studies investigating TNF effects on pituitary hormone secretion suggest that the proximity of cell types in slice cultures, in contrast to dispersed cultures, may affect hormone responses to stimuli *in vitro* (26).

As reviewed by Blalock (1), there is extensive cross-talk between the immune and endocrine systems. Because RBTNF was able to affect (decrease) the GH-releasing effect of GRF and TRH directly in pituitary slice cultures, our data suggest that TNF may be capable of modulating pituitary secretory capabilities by interacting directly with pituitary cells. Our data expand upon previous observations by demonstrating a significant interaction between RBTNF and GH-secretagogue mechanisms of GH release to modulate secretion. Furthermore, the demonstrations of bell-shaped dose-response curves associated with TNF *in vitro* (26) and the GH responses to TRH following increasing doses of RBTNF in this paper suggest that basal and stimulated GH secretion is impaired at high concentrations of TNF.

The presence of receptors specific for TNF on pituitary membranes, which has not been reported previously, supports this contention. Specific receptors for TNF in other tissues have been reported previously for other species (27–28). Our present findings suggest an affinity similar in magnitude to that reported by Pang *et al.* (29) in rat thyroid tissue. However, across several studies, there is a discrepancy in the affinity reported. Techniques such as the 4 *M* $MgCl_2$ have been used in other hormone-receptor binding studies, presumably to release endogenously bound ligand (30, 31). The net effect has usually been a generalized increase in total binding, an event that was noted in these studies with the TNF receptor. Although Kelly *et al.* (30) demonstrated that the affinity of the prolactin receptor was not affected by Mg^{2+} treatment, seldom are data presented that compare the effect of the Mg^{2+} treatment with no pretreatment in other hormone systems. Data in these studies suggest that the preincubation of the

membranes with 4 M $MgCl_2$ increased overall binding; however, the increase could be accounted for by the appearance of a low affinity-high capacity class of binding sites for TNF. The high affinity site in the two-site model and the single-affinity site measured in the non-treated preparations of pituitary membrane were of similar K_d value. Whether this phenomenon is related to the unmasking of hidden receptors or to an artifact of the chaotropic action of the Mg^{2+} treatment needs to be further investigated. Thissen *et al.* (31) suggested that the Mg^{2+} treatment of rat liver membranes resulted in a nonspecific loss of 20–30% of membrane proteins, necessitating an extrapolation to determine the number of total GH receptors. Since our protein contents of the membrane preparations were normalized following treatments, this nonspecific protein effect should not have influenced our findings. The overall results, when combined with the cross-reactivity binding data, are consistent with the concept that there are specific receptors for TNF-α in the pituitary. The relative sizes of membrane components that bind TNF were similar to those reported in L-M cells by Kull *et al.* (32) and reviewed by Jones *et al.* (28), with a major component residing in the region of 77,000 mol wt.

Several criteria supporting a role for TNF in modulating GH secretion have been satisfied. These include: (i) an action to alter basal hormone secretion, as demonstrated in an earlier study (11); (ii) the ability of the cytokine to interact with known GH regulatory mechanisms; (iii) the ability of RBTNF to alter characteristics of GH release into media under *in vitro* conditions, extensively removing the complications of whole body metabolic and cytokine influences; and (iv) the presence of specific saturable binding sited on the pituitary for TNF. We conclude, therefore, that TNF may affect pituitary GH secretion by direct interaction with specific receptors for TNF in the adenohypophysis and that TNF may function as a modulator of pituitary function. The data illustrate another level of control imposed on pituitary secretion by immune system components. These findings further impact on the nature of apparent pituitary dysfunction observed in different pathological situations in which a varied cytokine milieu is present.

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