Follistatin has a biphasic response but follicle-stimulating hormone is unchanged during an inflammatory episode in growing lambs

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Abstract

The effects on plasma follistatin concentrations of an inflammatory episode, induced by the intrathoracic injection of yeast, were examined in growing lambs; this model results in acute loss of appetite, food intake and liveweight and the activation of the acute-phase pathway for several weeks as adjudged by the production of haptoglobin and other acute-phase proteins. In these animals (n=8) there was a biphasic response in follistatin concentrations, with an initial 200% increase (P<0·001) in follistatin within 24 h of injection of yeast. Thereafter, follistatin concentrations were depressed to 70% of pretreatment levels 48 h after injection (P<0·01), followed by a gradual recovery of concentrations to pretreatment values. In another group of lambs (n=16) that were feed-restricted to mimic the reduced food intakes and liveweight changes in the yeast-injected group, plasma follistatin was also reduced to around 70% of pretreatment levels (P<0·01) within 1 day of the dietary regimen being implemented, followed by a gradual return to pretreatment values as food intakes were increased. Plasma follistatin correlated significantly (r=0·57, P<0·0001) with food intake, but not with liveweight changes. Plasma follistatin concentrations were unchanged in a third group fed *ad libitum* (n=8), except during two periods when food intakes were significantly (P<0·05) reduced, when follistatin concentrations also decreased (P<0·01). Plasma follicle-stimulating hormone (FSH) concentrations in the three groups of lambs were not significantly affected by the treatment regimes or changes in follistatin concentrations. These findings indicate that peripheral follistatin concentrations are modulated by both inflammatory and nutritional mechanisms, and that significant fluctuations in follistatin levels can occur without detectable perturbations in FSH secretion.

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Introduction

Follistatin is a glycoprotein that is found in a diverse range of tissues and has a multiplicity of functions during embryogenesis, development and adult life. It was isolated and characterized on the basis of its ability to inhibit the secretion of follicle-stimulating hormone (FSH) (Esch *et al.* 1987, Robertson *et al.* 1987, Ueno *et al.* 1987), and subsequently found to be a high-affinity binding protein of activin (Nakamura *et al.* 1990, Kogawa *et al.* 1991, Shimonaka *et al.* 1991, Schneyer *et al.* 1994), a member of the transforming growth factor-β superfamily.

Recently it has become apparent that follistatin is linked with cytokines involved in the generation of the acute-phase response and inflammation, namely interleukin-1 and tumour necrosis factor-α (TNFα). When interleukin-1β was injected into castrated rams, plasma follistatin concentrations increased robustly within 4 h, but had returned to pretreatment values within 24 h (Phillips *et al.* 1996). Using a similar approach, Klein *et al.* (1996) also reported that plasma follistatin concentrations increased following the activation of the inflammation pathway by injection of lipopolysaccharide, a bacterial cell wall component, into ewes. What is unclear at present is the effects of more extended inflammation on circulating follistatin concentrations, particularly whether follistatin is part of the classical acute-phase pathway generated in the liver (Baumann & Gauldie 1994) or part of a different response mechanism.

In the present report, we investigate the response of follistatin concentrations in a model where extended inflammation is induced in growing lambs by the intrathoracic injection of yeast (Pfeffer *et al.* 1993, Moore *et al.* 1995). In this model, the concentrations of a number of acute-phase proteins, such as haptoglobin, ceruloplasmin and fibrinogen, are elevated for up to 30 days (Pfeffer *et al.* 1993), and the animals suffer loss of appetite and reduced liveweight gain in the first few days after the injection of yeast. To account for the effects of changing nutritional status in yeast-injected animals, we also examined

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follistatin concentrations in animals that received a reduced ration of feed to mimic the weight loss induced by yeast injection.

Materials and Methods

Animals and experimental design

The experimental procedures reported in this study were carried out in accordance with the 1987 Animal Protection (Codes of Ethical Conduct) Regulations of New Zealand after approval was granted by the Animal Ethics Committee of the Wallaceville Animal Research Centre.

The details of the animals and treatments have been published previously (Moore *et al.* 1995). Briefly, 32 Romney ewe lambs were divided by stratified randomization at 5 months of age into three groups with similar weights, and housed indoors in individual pens. Each animal was prepared for injection by clipping the fleece on the right abdominal wall and soaking the skin with an alcohol solution. Those animals in the *ad libitum*-fed group (n=8) and the restricted-feeding group (n=16) were injected intrathoracically with 10 ml sterile non-pyrogenic saline solution, whereas the third group (n=8) were injected with 2·5 g baker's yeast (Tucker Group, Auckland, New Zealand) suspended in 10 ml non-pyrogenic saline solution. The animals in the *ad libitum*-fed and yeast-injected groups were offered 3·5 kg pelleted food each day, with the portion of the ration left each day being weighed to determine feed intakes. The animals on the restricted feeding regime were fed a ration designed to keep their weights similar to those in the yeast-injected group.

On predetermined days, the lambs were weighed and a blood sample collected by jugular venepuncture into vacutainer tubes containing EDTA (Becton and Dickinson, Rutherford, NJ, USA). Blood samples were centrifuged within 30 min of collection and the plasma stored at − 20 °C until they were assayed.

Hormone assays

All samples were assayed for follistatin using an RIA described previously (Klein *et al.* 1993, Phillips *et al.* 1996). This assay measures both the free and bound populations of follistatin by the use of dissociative agents in the assay. The standard employed was a purified preparation isolated from bovine follicular fluid (Robertson *et al.* 1987), and the antibody was one raised in a rabbit as described by Klein *et al.* (1993). In three assays, the assay sensitivity was <2·2 ng/ml, the mean ED_{50} was 7·5 ng/ml and the intra- and interassay coefficients of variation were 7·8 and 14·5% respectively. A random selection of plasma samples were run at several dilutions and found to run parallel to the standard curve.

FSH in plasma samples was measured in duplicate using a homologous RIA kit supplied by The National Hormone and Pituitary Program, as described previously (McNatty *et al.* 1989). The ovine (o) FSH used for iodination was NIDDK-oFSH-I-2, the reference preparation was NIDDK-oFSH-RP-2, and the oFSH antiserum was NIDDK-anti-oFSH-1. The intra- and interassay coefficients of variation were both <10% and the assay sensitivity was 0·2 ng/ml.

Data analyses

The effects of the treatment regimes were evaluated using repeated-measures ANOVA, with Dunnett's test used to compare the concentrations at day 0 with all other time points. To account for variations between untreated animals in the concentrations of follistatin (range 5·4–16·7 ng/ml), follistatin was also expressed as a percentage of the concentration at the time of injection (day 0). Correlation analyses were performed using the Pearson Product-Moment Correlation Coefficient and regression lines were generated using the least-squares method.

Results

Ad libitum-*fed group*

Growing lambs offered an above-maintenance ration had feed intakes that were relatively constant, with an overall mean of 47·6 g/kg liveweight per day. On two occasions after the start of the treatment, there was a significant (P<0·01) reduction in food intake, day 2 and day 9 (Fig. 1). Plasma follistatin concentrations were also relatively constant across the experimental period, with the exception of two sampling days when there was a significant (P<0·01 and P<0·05) reduction in mean follistatin concentrations, corresponding to those periods when food intake was lower. Plasma FSH concentrations were not significantly changed across the experimental period.

Yeast-injected group

Within 1 day of the yeast injection, appetite in this group was significantly depressed, with significantly (P<0·01) lower feed intakes between days 1 and 6 (Fig. 1). Thereafter, food intake recovered, and was not different from pretreatment values on days 7–17, except day 9, when there was a significant (P<0·01) reduction in intake compared with day 0. Plasma follistatin concentrations increased to more than 200% (P<0·001) of control values on day 1 after yeast injection. Thereafter, they were significantly reduced to around 70% (P<0·01) from day 2 to day 8, coinciding with the period of reduced feed intake

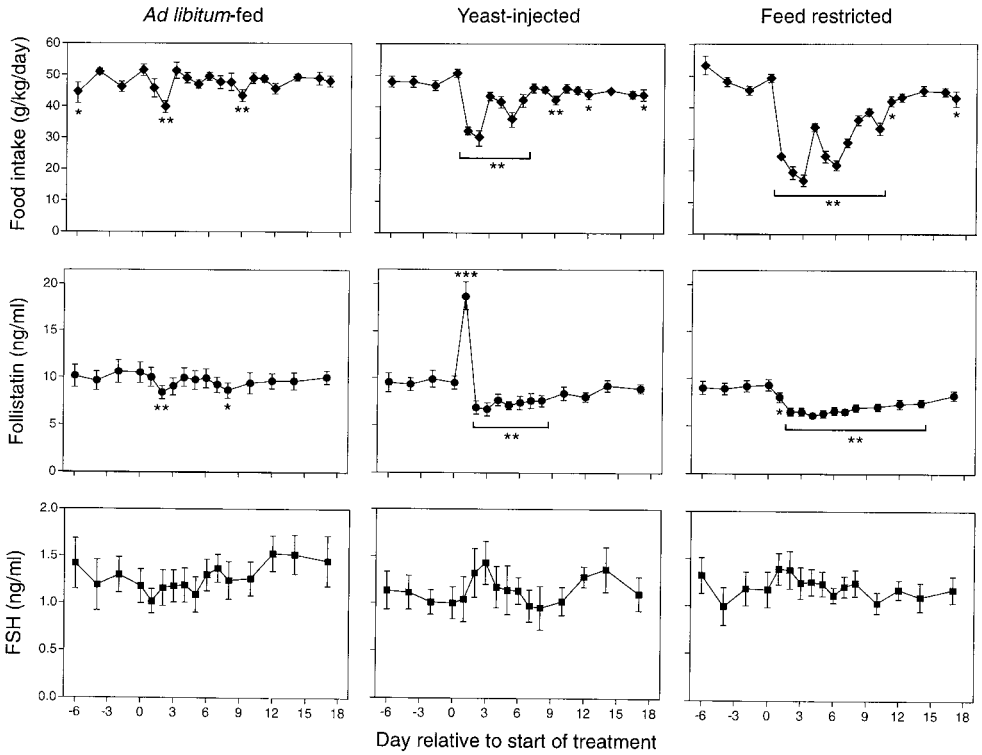

Figure 1 Mean (± S.E.M.) food intake (top panels), plasma concentrations of follistatin (middle panels) and FSH (bottom panels) in growing lambs fed *ad libitum*, injected intrathoracically with yeast, or under feeding restrictions to mimic the liveweight changes experienced by the yeast-injected group. Asterisks indicate significant differences within a parameter compared with values from day 0, using repeated-measures ANOVA (*P<0·05, **P<0·01, ***P<0·001).

in this group. Despite these dramatic changes in follistatin concentrations, there was no significant (P>0·05) change in plasma FSH concentrations.

Feed-restricted group

This group was offered a reduced food ration to mimic the feed intake and subsequent reduction in weight gain of those animals in the yeast-injected group. Food intake was significantly (P<0·01) depressed on days 1–11, and thereafter was similar (P<0·05) to pretreatment values (Fig. 1). In terms of follistatin concentrations, there was a significant (P<0·05) reduction within 1 day of feed restrictions being introduced, and the levels remained at around 70% of control values (P<0·01) on days 2–14. The changes in follistatin occurred independently of FSH concentrations, which were largely unchanged during this period. No correlation was observed between FSH and follistatin concentrations in this group or in the other two experimental groups (data not shown). Food intake and follistatin concentrations correlated significantly (P<0·05) in 12/16 animals in this group. When food intake and plasma follistatin concentrations were plotted (Fig. 2A), the correlation coefficient was r=0·57, with a significance level of P<0·0001. In contrast, plasma follistatin concentrations in this group were not related to changes in liveweight (Fig. 2B).

Discussion

The key finding from this study is a biphasic response of peripheral follistatin concentrations to the generation of a chronic inflammatory episode: an initial increase in follistatin within the first 24 h after the initiation of an acute-phase response, followed by a longer suppression of follistatin concentrations coinciding with the period of depressed appetite and food intake. We and others have documented previously the initial phase of increased follistatin concentrations in both castrated rams treated with interleukin-1β (Phillips *et al.* 1996) and ovariectomized ewes given lipopolysaccharide (Klein *et al.* 1996). In both these models, plasma TNFα concentrations rose within 1 h of injection of the acute-phase inducers, but remained elevated for only a few hours after the initiation of an inflammatory episode. In the model used in the present study, a number of acute-phase proteins such as haptoglobin, ceruloplasmin and fibrinogen are elevated for

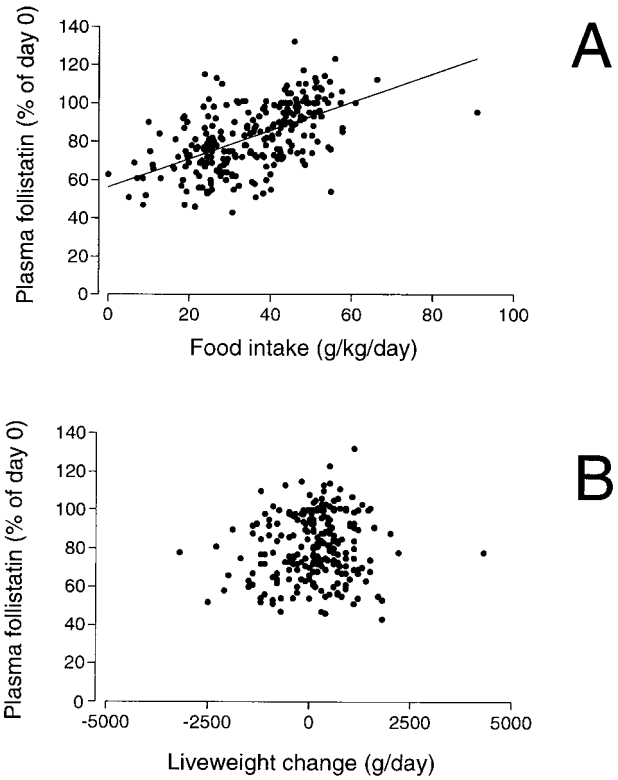

Figure 2 (A) The relationship between plasma follistatin (expressed as the percentage of the concentration relative to the start of treatment) and feed intake in the feed-restricted group (n=16 animals); r=0·57, P<0·0001. The line of best fit has a slope of 0·73 ± 0·07, the y-intercept is 56·5 ± 2·5 and the x-intercept is − 77·2. (B) Lack of a relationship between plasma follistatin and liveweight changes in the feed-restricted group.

up to 30 days after intrathoracic injection of yeast (Pfeffer *et al.* 1993). As follistatin concentrations were elevated for only the first 24 h in the face of chronic inflammation, this suggests that the increase in follistatin is mediated by one or more of the initial components of the pathway. This thesis is supported by recent findings showing that follistatin mRNA is up-regulated within 4 h of exposure of cultured porcine endothelial cells to lipopolysaccharide (Michel *et al.* 1996). Furthermore, since follistatin concentrations are elevated for only 24 h in the present study whereas a number of classical acute-phase proteins in this model are elevated for up to 20 days (Pfeffer *et al.* 1993, Moore *et al.* 1995), it seems unlikely that the release of follistatin is part of the acute-phase pathway centred in the liver, where acute-phase proteins are synthesized and released (Baumann & Gauldie 1994). However, direct evidence as to whether follistatin is an acute-phase protein as defined by hepatic release under the influence of inflammatory mediators is not currently available and this needs to be explored further.

The mechanism(s) by which follistatin is up-regulated by the inflammatory cascade remains obscure, but most probably involves the cytokines interleukin 1 and/or TNFα. The *in vivo* data presented herein and in previous studies (Klein *et al.* 1996, Phillips *et al.* 1996) are strongly supportive of this proposal, and the study of Michel *et al.* (1996) using cultured vascular endothelial cells treated with lipopolysaccharide is also consistent with a role for these cytokines as secretagogues for follistatin. Endothelial cells are known to produce interleukin-1 and TNFα under stimulation from lipopolysaccharide (Corsini *et al.* 1996), which could explain why *in vivo* models utilizing injection of lipopolysaccharide (Klein *et al.* 1996), yeast (present study) or interleukin-1β (Phillips *et al.* 1996) all gave similar follistatin-response profiles. Although direct effects of interleukin-1β and TNFα on follistatin production have not been documented, inhibin and activin, to which follistatin can act as a binding protein, are modulated by these agents. In cultured rat granulosa cells, both interleukin-1β and TNFα can inhibit the production of inhibin under the influence of FSH (Imai *et al.* 1996). On the other hand, interleukin-1β and TNFα stimulate the activin β_A subunit mRNA in cultured mouse fibroblasts and keratinocytes (Hübner & Werner 1996).

Follistatin mRNA is found in many tissue and cell types, both of gonadal and extragonadal sources (Michel *et al.* 1990, 1996, Kaiser *et al.* 1992, Tuuri *et al.* 1994). From recent work illustrating that vascular endothelial cells can rapidly up-regulate follistatin mRNA under the influence of lipopolysaccharide (Michel *et al.* 1996), a major source of circulating follistatin after the induction of an inflammatory episode is likely to come from vascular tissues. This does not preclude that other tissue or cellular sources are regulated in a similar manner under the influence of an acute-phase response. Further studies in an *in vivo* model of inflammation are needed to establish which tissue reservoirs are responding by synthesizing and secreting follistatin into the circulation.

A novel finding of this study is that follistatin concentrations are affected by nutritional status; plasma follistatin correlated positively with feed intake. Moreover, plasma follistatin concentrations decreased within 1 day of reduced feeding, and were also apparent in the *ad libitum*-fed group on the 2 days when feed intakes were significantly lower. The mechanism(s) responsible for this correlation is unclear, and we are currently exploring this phenomenon in further detail. Of note is that activin A is known to stimulate glucose production in cultured rat hepatocytes (Mine *et al.* 1989, 1996), although this glycogenolytic activity is attenuated in the intact rat liver (Kojima *et al.* 1995). With regard to inhibin, another member of the transforming growth factor-β superfamily, nutritional status does not appear to be a major influence on plasma inhibin concentrations in male rats (Dong *et al.* 1993) and sheep (Martin *et al.* 1994). Interestingly, in inhibin α-subunit-deficient mice, where activin concentrations are extremely high, there are marked effects on the mucosal layer of the glandular stomach and an absence of

parietal cells (Matzuk *et al.* 1994, Coerver *et al.* 1996). Whether this observation is related to the effects of food intake on follistatin concentration in the present study remains to be determined.

Of interest in the present study is the finding that changes in follistatin concentrations caused by the experimental treatments were not accompanied by any significant changes in FSH concentrations or correlation between follistatin and FSH. Follistatin was originally characterized as a suppressor of FSH concentrations (Esch *et al.* 1987, Robertson *et al.* 1987, Ueno *et al.* 1987), and injection of pharmacological amounts of follistatin into a number of animal species has confirmed that follistatin can lower peripheral FSH concentrations (dePaolo *et al.* 1991*a*, Inouye *et al.* 1991, Meriggiola *et al.* 1994, Tilbrook *et al.* 1995). Prepubertal lambs are known to be responsive to feedback effects of the known regulators of FSH such as steroids and inhibin (Pelletier *et al.* 1981). Nevertheless, a number of studies in which follistatin and FSH concentrations have been measured suggest that follistatin is not a potent regulator of peripheral FSH levels in several endocrine conditions (Klein *et al.* 1993, Gilfillan & Robertson 1995, Khoury *et al.* 1995). The primary role of follistatin in regulating FSH may be achieved through a paracrine/autocrine interaction with activin, as has been proposed in the pituitary gland (dePaolo *et al.* 1991*b*, Mather *et al.* 1993, Bilezikjian *et al.* 1993, 1996, Halvorson *et al.* 1994).

In summary, plasma follistatin concentrations in yeast-injected lambs showed a biphasic response, with an initial increase in concentrations under an inflammatory mechanism, followed by an inhibitory phase induced by reduced nutrition. The follistatin concentrations correlated positively with food intake but not liveweight changes, and were not related to plasma FSH concentrations. These findings suggest that peripheral follistatin levels may be regulated through several mechanisms not related to the 'classical' role of follistatin as an FSH inhibitor, through its ability to neutralize the stimulatory action of activin on FSH secretion.

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