Active Immunization against Adrenocorticotropic Hormone in Growing-Finishing Barrows: An Initial Trial and Evaluation

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ABSTRACT : Adrenal glucocorticoids, secreted by the stimulus of adrenocorticotropic hormone (ACTH), are catabolic hormones in the pig. The present study was conducted to find whether active immunization against ACTH would suppress cortisol secretion accompanied by an increased growth rate in growing-finishing barrows. ACTH was conjugated to keyhole limpet hemocyanin or human histone using glutaraldehyde or 3-maleimidobenzoic acid *N*-hydroxysuccinimide, under a 2 (ACTH vs no hapten)×2 (carrier)×2 (crosslinker) factorial arrangement of treatments. Cross-bred barrows weighing approximately 25 kg were injected with an ACTH-carrier or carrier only conjugate every 4th wk and slaughtered at approximately 110 kg body weight. Antibodies against ACTH were detected in serum, as determined by [^{125}I]ACTH-binding activity, in most animals immunized against the ACTH conjugate, but not in carrier only-injected animals, except for the animals which had received the hapten conjugated to histone via glutaraldehyde. The [^{125}I]ACTH-binding activity of serum increased after the second booster injection, but overall ACTH antibody titer was very low. Main effect was not detected not only for the carrier and crosslinker but for the hapten in serum cortisol concentration, ADG, loin muscle area, backfat thickness and longissimus muscle composition including fat and protein. In addition, bound [^{125}I]ACTH percentage had no relation to cortisol concentration or to any of the above growth-related variables. Results suggest that ACTH or its conjugates used in the present study were not immunogenically potent enough to affect the glucocorticoid secretion and thus the growth of the immunized pigs. ***(Asian-Aust. J. Anim. Sci. 2002. Vol 15, No. 3 : 410-415)***

Key Words : ACTH, Cortisol, Active Immunization, Growth, Barrow

INTRODUCTION

Growth of farm animals is regulated by an array of hormonal actions (Etherton and Kensinger, 1984). Accordingly, attempts to manipulate animal growth have been focused on altering circulating concentrations of those hormones that are related to growth (Beermann, 1989). These include injection of somatotropin, oral administration of β-adrenergic agonists, implantation of anabolic steroids and immunoneutralization of growth-related hormones, etc.

Adrenocorticotropic hormone (ACTH), which is secreted from pituitary by the stimulus of corticotropin-releasing factor and vasopressin (Antoni, 1989), is a 39-amino-acid peptide (Voigt et al., 1990) that stimulates the secretion of adrenal glucocorticoids (Minton and Parsons, 1993). The ACTH and glucocorticoids are catabolic hormones by nature in the pig and rat, as evidenced by their negative effects on growth in these species (Chapple et al., 1989; Guo et al., 2000; Huang et al., 2000). Moreover, Sillence et al. (1992) have reported that growth rate was increased by a passive immunization against ACTH in the rat, suggesting the possibility that animal growth may be enhanced by active immunization against this peptide. To authors' knowledge, however, such a possibility has not been examined to date. The present study was therefore undertaken to investigate the effect of active immunization against ACTH on circulating cortisol concentration and growth in the pig and thereby to find insights into the possibility of this method as a means of growth manipulation.

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MATERIALS AND METHODS

Antigen preparation

Porcine adrenocorticotropic hormone (ACTH) extracted from the pituitary (Sigma Chemical Co., Saint Louis, MO, USA) was conjugated to keyhole limpet hemocyanin (KLH) or human histone carrier using either 3-maleimidobenzoic acid *N*-hydroxysuccinimide (MBS) or glutaraldehyde, under a 2 × 2 × 2 factorial arrangement of treatments; main effects included hapten (ACTH vs none), carrier and crosslinker. The hapten-carrier conjugation using MBS was performed following the procedure of Liu et al. (1979). Briefly, 70 mg KLH or histone dissolved in 4.4 ml of 50 mM phosphate-buffered saline (PBS), pH 7.4, was mixed with 875 μl of 6.0 mg MBS/ml *N*, *N*-dimethyl formamide solution for 30 min on a rocker at room temperature. After removing free MBS on a P-6 disposable gel filtration column (Bio-Rad, Hercules, CA, USA), fractions corresponding to activated carrier were pooled and mixed with 13.2 ml PBS containing 70 mg dissolved ACTH or none for 3 h on a stirring plate. The hapten-carrier

conjugate was dialyzed (1,000 of MW cut-off) against 1 L PBS three times at 12 h intervals, diluted to 40 ml with PBS, divided into five aliquots and stored at -70°C until used.

The hapten-carrier conjugation using glutaraldehyde was performed as described by Harlow and Lane (1988). The ACTH hapten (70 mg) was conjugated to an equal amount of KLH or histone in 15 ml PBS by drop-wise addition of an equal volume of 0.2% glutaraldehyde on a stirring plate for 1 h in a fume hood. The conjugation reaction was terminated with 7.5 ml of 1 M glycine in PBS, pH 7.2, for 1 h on a stirring plate, followed by dialysis as described above.

Animals

Sixty-four barrows weighing approximately 25 kg, which were progenies of Landrace×Yorkshire sires×Duroc dams, were randomly divided into eight pens in such a way that each group of the hapten-carrier-crosslinker combination was assigned eight animals in one pen. Each animal received a 1 ml intramuscular injection of the immunogen mixed with an equal volume of Freund's complete adjuvant at two sites of the neck at the dorsal side, followed by booster injections with Freund's incomplete adjuvant at 4 wk intervals. Blood samples were taken from jugular vein (Yun et al., 2001) 2 wk after each booster injection. The animals were fed *ad libitum* commercial diets as follows: a weaner containing 18% CP and 3.35 Mcal DE/kg up to 35 kg body weight, a grower (15.6% CP and 3.33 Mcal DE/kg) up to 55 kg and thereafter a finisher (15.5% CP and 3.29 Mcal DE/kg). Animals weighing approximately 110 kg irrespective of the pen were transported to a local slaughterhouse located within 1.5 h transportation and slaughtered the following day. This procedure was repeated four times approximately at 1 wk intervals to slaughter all the animals within a narrow range of live weight. Loin muscle area (LMA) was estimated at the 10th rib using an ultrasound scanner (model SSD-500V, Aloka Co., Tokyo, Japan) prior to transportation for slaughter; backfat thickness was measured after overnight chilling of the carcass at 4°C at the last rib and between the 11th and 12th ribs. The estimated LMA and the average backfat thickness were adjusted for 110 kg live weight using the following equations suggested by the National Swine Improvement Federation (NSIF, 1997, USA) for barrows. Adjusted LMA=actual LMA+[(desired wt-actual wt)×actual LMA÷(actual wt+70.31)]; adjusted backfat= actual backfat+[(desired wt-actual wt)×actual backfat÷(actual wt-13.608)], where wt was in kilograms.

Chemical analysis of longissimus muscle

Whole longissimus muscle dissected from a chilled half-carcass per each animal was ground and an aliquot was stored in a sealed plastic bag at -70°C until analyzed. Moisture, protein and fat were determined by freeze-drying, Kjeldahl method and Soxhlet extraction, respectively, following the procedure of AOAC (1990).

Antibody titering and cortisol assay

The ACTH (0.5 μg) was iodinated to a specific activity of 150 μCi/μg using 0.3 mCi $Na^{125}I$ (Amersham Pharmacia Biotech, Arlington Heights, IL, USA) and chloramine-T as previously described (Lee and Henricks, 1990). Four microliters of serum were incubated with 30,000 cpm [^{125}I]ACTH in 0.2 ml (total volume) PBS, pH 7.4, overnight at 4°C in 0.5 ml microfuge tubes. Bound [^{125}I]ACTH was precipitated by incubation with 50 μl of 10% protein A-agarose (Life Technologies, Gaithersburg, MD, USA) in PBS on a rotating mixer overnight at 4°C, followed by centrifugation at 13,000 rpm for 5 min at 4°C, removal of supernatant and γ-counting. Non-specifically bound (NSB) count, which was defined as the count of the wk 0 preimmune serum in each animal, was subtracted from the total bound count. For a comparison of the titer of the ACTH antisera with that of a known antiserum, specific binding of 30,000 cpm [^{125}I]IGF-I to a commercial IGF-I antiserum (Gropep, Adelaide, Australia) diluted at 1:4,000 in the presence of 4 μl wk 0 preimmune serum also was measured.

Serum cortisol concentration was determined using a RIA kit (Diagnostic Products Corporation, Los Angeles, CA, USA) as previously described by Yoon et al. (2001).

Statistical analysis

Measurements of the live animal and carcass, bound [^{125}I]ACTH percentage and serum cortisol concentration were analyzed using the GLM procedure of SAS (1986). The model included main effects (hapten, carrier and crosslinker), two-way interactions of these and a three-way interaction. Also included in the model for the repeated measurement were blood sampling wk and corresponding interactions associated with it. When no main effect was detected, data were also analyzed using a reduced model containing only the immune response (negative vs positive) with or without the blood sampling wk. In all the analyses, animal nested within hapten×carrier×crosslinker or the immune response, which was the experimental unit, was used as the error term for the test of significance.

RESULTS

The [^{125}I]ACTH bound specifically to sera taken from animals immunized against an ACTH conjugate following the first booster immunization (6 wk after the initial immunization), but not to sera from those immunized against the carrier only. Moreover, bound [^{125}I]ACTH percentage increased with decreasing dilution of serum

between 1:2,000 and 1:50 (data not shown). Six out of six glutaraldehyde/KLH group animals, five out of seven MBS/KLH animals and all the seven MBS/histone group animals exhibited the [^{125}I]ACTH-binding activity; for unknown reason(s), however, [^{125}I]ACTH binding was not detected at any time point in animals immunized against the hapten that had been conjugated to human histone using glutaraldehyde (figure 1). The bound [^{125}I]ACTH percentage increased between 6 and 10 wk after the second booster injection, but not between 10 and 14 wk after the third booster immunization.

Growth performance and chemical composition of longissimus muscle of the animals are shown in table 1. Average daily gain (ADG) was slightly greater ($p<0.05$) in the glutaraldehyde group than in MBS group. However, effects of hapten and carrier on ADG as well as all the three main effects, i.e. hapten, carrier and crosslinker, on final weight, carcass weight, dressing percentage and backfat thickness and loin muscle area adjusted for 110 kg body weight were not significant ($p>0.05$). Moisture content of longissimus muscle was greater in the ACTH hapten group compared with no hapten control (72.7±0.5 vs 71.6±0.4%) when KLH was used as carrier, whereas the reverse was true (70.9±0.5 vs 72.2±0.5%) with histone carrier ($p<0.05$). However, main effects of hapten, carrier and crosslinker on moisture, fat and protein contents of longissimus muscle were not significant ($p>0.05$). Interactions between main effects, except for the aforementioned carrier×hapten in longissimus muscle moisture content, also were not significant in any of these variables. Effects of the immunization were further analyzed using a reduced model containing only the immune response which had been determined according to the presence or absence of serum [^{125}I]ACTH-binding activity. No difference was detected between the ACTH antibody-positive and -negative groups in any of the variables related to growth and longissimus muscle composition (table 1). Moreover, neither body weight nor serum cortisol concentration differed between the two groups at any time when body weight was measured and the blood sample was taken (figure 2); ADG also did not differ between the two groups during any interval (data not shown).

Shown in table 2 are correlations between variables related to growth and longissimus muscle composition in total animals and the relationship of bound [^{125}I]ACTH

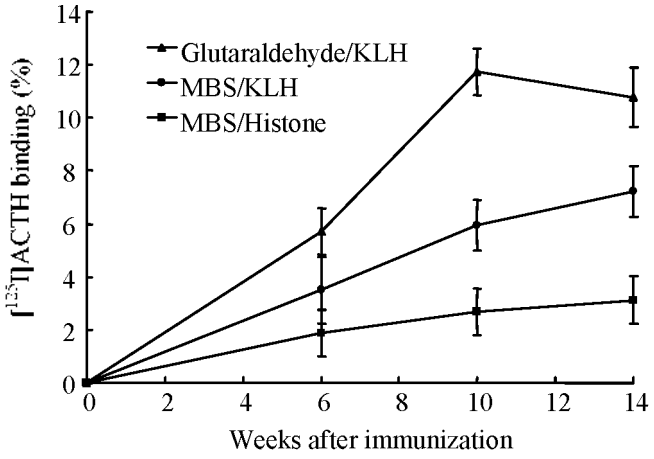

Figure 1. Serum [^{125}I]ACTH-binding activity of the animals actively immunized against ACTH. The ACTH hapten that had been conjugated to keyhole limpet hemocyanin (KLH) or human histone using glutaraldehyde or 3-maleimidobenzoic acid *N*-hydroxysuccinimide (MBS) was injected at wk 0 of the experiment, followed by booster injections at wk 4, 8 and 12. Serum (4 μl) taken at the indicated time was incubated with 30,000 cpm [^{125}I]ACTH in 0.2 ml PBS overnight at 4°C, followed by precipitation of bound [^{125}I]ACTH using protein A-agarose. The [^{125}I]ACTH-binding activity was not detected in carrier only-injected animals or in animals injected with the ACTH hapten that had been conjugated to histone via glutaraldehyde. Data are LS means±SE of bound [^{125}I]ACTH percentage. Under the present assay condition, a commercial IGF-I antiserum diluted at 1:4,000 bound >90% [^{125}I]IGF-I.

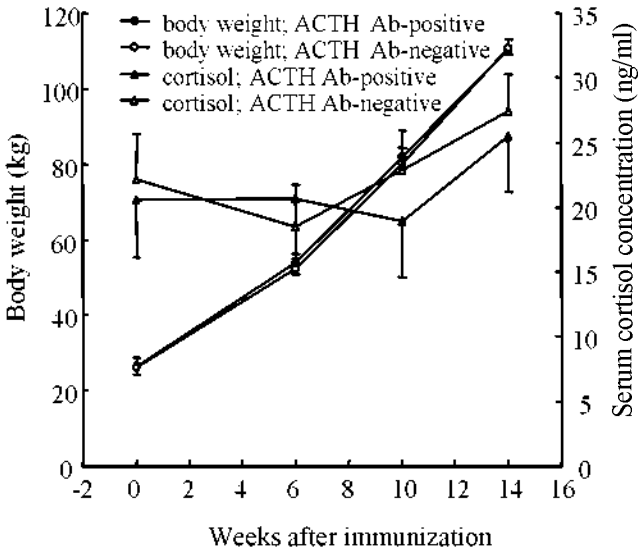

Figure 2. Body weight and serum cortisol concentration of ACTH antibody-positive and -negative barrows after active immunization against the hapten-carrier conjugate or the carrier only. The presence of ACTH antibodies was determined according to the [^{125}I]ACTH-binding activity of serum (figure 1). Data are LS means + or - SE. Neither overall mean cortisol concentration nor overall ADG differed ($p>0.05$) between the two groups.

Table 1. Effects of active immunization against ACTH on growth and chemical composition of longissimus muscle in cross-bred growing-finishing barrows

Items	Hapten[a]		Carrier[a]		Crosslinker[a]		ACTH antibody[b]	
	None	ACTH	KLH	Histone	MBS	Glutaraldehyde	Negative	Positive
Initial wt. kg	26.2±0.6	25.7±0.7	26.2±0.7	25.6±0.6	26.7±0.6	25.2±0.7	26.3±0.6	25.9±0.9
Final wt. kg	113.2±1.0	110.7±1.0	112.0±1.0	111.9±1.0	112.2±0.9	112.7±1.0	112.6±0.8	109.9±1.3
ADG. kg	0.83±0.01	0.82±0.02	0.83±0.02	0.82±0.02	0.80±0.01	0.85±0.02*	0.82±0.01	0.81±0.02
Carcass wt. kg	81.6±0.8	79.8±0.8	81.0±0.8	80.4±0.8	80.2±0.8	81.2±0.8	81.1±0.7	79.6±1.0
Dressing. %	75.2±1.5	78.4±1.6	77.6±1.6	76.0±1.6	77.5±1.5	76.1±1.6	76.8±1.3	76.4±2.0
Backfat thickness[c], mm	20.9±0.7	20.7±0.7	20.6±0.7	21.1±0.7	20.6±0.7	21.0±0.8	20.4±0.6	21.9±0.9
LMA[d], cm^2	35.7±1.1	35.4±1.1	34.5±1.1	36.6±1.1	36.0±1.0	35.2±1.1	35.9±0.9	35.3±1.4
Chemical composition[e]								
Moisture. %	71.9±0.3	71.8±0.4	72.2±0.3	71.6±0.3	71.9±0.3	71.9±0.3	71.7±0.3	72.1±0.5
Fat. %	4.6±0.3	4.3±0.4	4.2±0.4	4.7±0.4	4.4±0.4	4.4±0.4	4.7±0.3	4.0±0.5
Protein. %	21.0±0.1	21.0±0.1	21.1±0.1	21.1±0.1	21.1±0.1	21.0±0.1	21.0±0.1	21.1±0.2

[a] ACTH hapten was conjugated to keyhole limpet hemocyanin (KLH) or human histone using either 3-maleimidobenzonic acid *N*-hydroxysuccinimide (MBS) or glutaraldehyde. Interactions between treatments were non-significant, except for a carrier×hapten interaction in moisture percentage which is described in detail in the text. Data are LS means±SE.

[b] The presence of ACTH antibodies in serum was determined according to the [^{125}I]ACTH-binding activity. The model included only the presence or absence of ACTH antibodies. Data are LS means±SE.

[c] Average thickness adjusted for 110 kg live weight at the last rib and between the 11th and 12th ribs.

[d] Loin muscle area, adjusted for 110 kg body weight, estimated by real-time untrasound scanning.

[e] Chemical composition of the whole longissimus muscle.

* $p<0.05$ within the treatment.

Table 2. Pearson's correlations between variables related to growth and longissimus muscle composition, serum cortisol concentration and bound [^{125}I]ACTH percentage in cross-bred barrows actively immunized against the conjugated ACTH or the carrier only

	ADG		Backfat[a]		LMA[b]		Moisture[c]		Protein[c]		Fat[c]		Cortisol	
	r	p	r	p	r	p	r	p	r	p	r	p	r	p
Total animals (n=57)														
ADG	-		0.06	0.67	-0.49	< 0.01	0.21	0.16	-0.23	0.13	-0.20	0.18	-	
Backfat	-		-		-0.03	0.82	0.07	0.63	-0.09	0.53	-0.11	0.46	-	
LMA	-		-		-		-0.34	0.02	0.17	0.25	0.14	0.37	-	
Moisture	-		-		-		-		-0.05	0.75	-0.54	<0.01	-	
Protein	-		-		-		-		-		-0.01	0.94	-	
ACTH antibody-positive animals (n=18)[d]														
Wk 6	-0.05	0.87	0.23	0.45	0.26	0.40	-0.21	0.53	0.24	0.47	-0.12	0.72	-0.39	0.15
Wk 10	0.17	0.56	-0.07	0.79	0.00	0.99	0.12	0.70	-0.11	0.72	0.13	0.68	0.16	0.56
Wk 14	0.19	0.50	-0.15	0.60	-0.03	0.92	0.11	0.72	0.03	0.92	0.04	0.90	-0.05	0.84

[a] Average thickness adjusted for 110 kg live weight at the last rib and between the 11th and 12th ribs.

[b] Loin muscle area, adjusted for 110 kg body weight, estimated by real-time ultrasound scanning.

[c] Percent content of each component of the whole longissimus muscle.

[d] The presence of ACTH antibodies in serum was determined according to the [^{125}I]ACTH-binding activity. Data denote correlations between the variable in each column and bound [^{125}I]ACTH percentage at the indicated time after the initial immunization.

percentage to above variables and serum cortisol concentration in ACTH antibody-positive animals only. In total animals, moisture content of longissimus muscle was, as expected, negatively correlated with fat content. It also was noteworthy that loin muscle area adjusted for 110 kg live weight was negatively correlated with ADG and moisture content of the muscle. Correlations between other growth variables and chemical components, however, were not significant. In ACTH antibody-positive animals, neither serum cortisol concentration nor other variables exhibited any correlation with the bound [^{125}I]ACTH percentage at any time point.

DISCUSSION

Adrenal glucocorticoids are generally known as catabolic hormones. Nevertheless, for unclear reason(s), administration of a glucocoricoid is not always accompanied by a reduced growth rate. For instance, dexamethasone, a potent synthetic glucocorticoid, has been reported to have no apparent effect on growth rate in cattle (Corah et al., 1995), whereas in the pig and rat, exogenous glucocorticoid including dexamethasone (Weiler et al., 1997; Guo et al., 2000; Huang et al., 2000) or ACTH (Chapple et al., 1989) has consistently been associated with a decreased growth rate. In addition, Sillence et al. (1992) reported a 37% increase in weight gain in passively immunized rats against ACTH. Taken together, these results suggest that the adrenocorticoid is primarily a catabolic hormone in the pig as well as in the rat. The present study was thus undertaken with an expectation that immunoneutralization of ACTH might result in an increase in weight gain and possibly lean mass in the pig.

Antibodies against ACTH were detected in most animals following the immunization against the antigen that had been conjugated either to KLH or human histone using MBS or to KLH using glutaraldehyde. However, the antibody titer of the animals that had responded to the immunogen in the present study was very low when compared with those in the animals immunized against a hypothalamic hormone (Adams and Adams, 1990; Armstrong et al., 1991; Guillaume et al., 1992). Specifically, the <12% [^{125}I]ACTH binding of the present antisera at 1:50 dilution was much less than the >50% binding at a comparable dilution of antisera or the 10 to 50% binding at 1:≥1,000 dilution of antisera in those studies. Thus, the lack of effect of the present active immunization on serum cortisol concentration and growth was probably related to the low antibody titer. Also noticeable in the present study was that ACTH antibodies were not detected in the animals that had received the antigen conjugated to human histone via glutaraldehyde. The lack of immune response in this group of animals is most likely to have resulted either from a poor conjugation efficiency or from an extremely poor immunogenecity of the conjugate, but neither possibility could be pursued in the present study because the ACTH antibody titering was performed after the injection of the last aliquot of the conjugated immunogen.

There are only a few speculations at the present as to why the immunized animals exhibited a low antibody titer. First, it seems likely that relative to well-known peptide immunogens, ACTH hapten itself or its conjugates used in the present study are only sparingly immunogenic. It has already been established that the immune responsiveness depends on the immungenecity of the hapten itself as well as carrier and crosslinker (Kim et al., 1998). Second, the amount of released ACTH antibodies in immunized animals may not have been enough to effectively neutralize the action of this episodically secreted hormone, although there is an incidence where gonadal function was successfully suppressed by an active immunization against a gonadotropin (Esbenshade et al., 1990). In this regard, immunization against a hypothalamic hormone may be more effective than that against its corresponding pituitary hormone, as has been exemplified in a study of active immunization against GnRH vs FSH or LH (Thau, 1992).

In conclusion, results of this initial trial suggest that the immunogenecity of ACTH or its conjugates used in the present study are not potent enough to suppress glucocorticoid secretion. Therefore, the present immunization regimen will not probably be effective for promoting growth of pigs. It will be worthwhile, however, to further investigate the effect of active immunization against corticotropin-releasing factor or ACTH conjugates different from those used in the present study.

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