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Passive Immunization Approach to Reduce Body Fat in Pigs Using Fat-specific Polyclonal Antiserum

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ABSTRACT : Plasma membrane proteins from pig adipocytes, brain, heart, kidney, liver and spleen were isolated using a 32% sucrose gradient. An adult male sheep was immunized three times at 3-wk intervals with the purified pig adipocyte plasma membrane (APM) proteins. Blood samples were taken from the immunized sheep 12 d after the third immunization. Antiserum showed strong reactivity with APM proteins determined by ELISA, and the reactivity could be detected at dilutions in excess of 1:128,000. Antiserum showed very low binding affinity with proteins isolated from brain, heart, kidney, liver or spleen. Ninety weanling pigs were allocated randomly to three treatment groups and were injected i.p. with 40 ml of antiserum (n = 30) or 20 ml of lyophilized antiserum (21.5 mg/ml; n = 30). A control group (n = 30) received 40 ml of saline, and all pigs were slaughtered at 24 wk of age. The polyclonal antiserum did not change BW or ADG. Carcass percentage of pigs was numerically increased by the antiserum treatment compared with control. Both antiserum treatments did not significantly ($p>0.05$) affect body composition, including body fat content, relative to the control group. (**Key Words :** Pig, Adipocytes, Polyclonal Antiserum, ELISA, Carcass Percentage, Body Composition)

INTRODUCTION

The reduction of fat deposition is of considerable common matter of interest. In actual fact, coronary arteries disease is the major cause of death in adult in advanced countries. There is, therefore, considerable interest in techniques that increase the efficiency of meat production by repartitioning energy from fat into protein deposition.

The development of approaches based on the use of exogenous substances principally involves steroid types and growth hormones, which have been successfully used world-wide (Williams, 1987; Moloney et al., 1990; Zhang and Grieve, 1995). However, increasing concerns over the use of such compounds from the view point of human safety led to a ban on their use in some countries especially in European Community (EC) (Moloney et al., 1989; Moloney, 1995). This ban encouraged researches into a wide variety of alternative techniques which were hoped to be more 'consumer favorable'.

With these direct and indirect backgrounds, many researchers were able to develop polyclonal antibodies raised against adipocyte plasma membranes (APM) in rat (Flint et al., 1986; Panton et al., 1990; Flint et al., 1998), rabbit (Dulor et al., 1990), bovine (Cryer et al., 1984), sheep (Moloney et al., 2002), pig (Kestin et al., 1993) and chicken (Butterwith et al., 1989) for body fat reduction in meat animals. The potential of generating antibodies that recognize specific APM proteins thought to be attractive to reduce body fat in meat animals. However, the referred studies as above have been focused on either production or cytotoxic effect of adipocyte specific serum, therefore, we have some unsolved problem (ex. *in vivo* cross-reactivity, status of antibody, concentration of antibody injected, immunization method and frequency, antibody delivery system, etc.) for the practical application of the results from these studies.

Thus, the object of this experiment was to produce polyclonal antibodies to APM proteins isolated from pig, to characterize the antibodies, and to investigate the tissue specificity. Finally, *in vitro* and *in vivo* cytotoxicity of the polyclonal antiserum was determined using primarily cultured pig adipocytes and piglets, respectively.

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MATERIALS AND METHODS

Isolation of PM proteins from various tissue of pig

Adipocyte : Plasma membrane (PM) proteins of adipocytes from pig were isolated according to the method of Kestin et al. (1993), and Vasilatos et al. (1983). Adipose tissue was obtained from pig (90-100 kg, male) slaughtered at a local abattoir. Adipose tissue samples were dissected free from connective tissue and blood vessels. The tissues were minced and incubated in digestion media (Medium 199 Hepes Modification (M2520, Sigma Chemical), 30% bovine serum albumin (BSA; A2153, Sigma Chemical), collagenase (1 mg/buffer; C6885, Sigma Chemical)) for 90 min. The infranatant containing immature adipocytes was discarded and then the remained mature adipocytes were collected. Adipocytes were lysed by shaking vigorously with hands in 2-3 volumes pre-warmed MEM (membrane extraction medium: 0.01 M Na_2HPO_4, 0.25 M sucrose, 2 mM EGTA, 200 ml distilled water, pH 7.4) and 0.2 M PMSF. The homogenate was centrifuged at 2,800 rpm for 5 min at 4°C to remove floating fat. The infranatant and pellet containing PM proteins were mixed and centrifuged at 35,000 rpm for 1 h at 4°C. After centrifugation, the pellet was resuspended in cold 32% sucrose extraction medium (0.25 M Sucrose, 10 mM Tris, 2 mM EGTA, pH 7.4). APM proteins were isolated using high speed spin (35,000 rpm, 1 h, 4°C). The proteins mixed with MEM solution were purified using high speed centrifugation (35,000 rpm, 1 h, 4°C).

Brain, heart, kidney, liver and spleen : PM proteins from brain, heart, kidney, liver and spleen were isolated according to the method of Lo et al. (1976). The frozen pig tissues of brain, heart, kidney, liver and spleen were minced. The tissues were homogenized with 30 ml MEM in a polytron homogenizer (Janke & Kunkel co., Staferin, Germany) at low setting for 2 min then at high setting for 2 min. The homogenized tissues were centrifuged at 3,000 rpm for 30 min at 4°C and then plunged into ice. The infranatant centrifuged at 35,000 rpm for 1 h at 4°C. After centrifugation, the pellet was suspended in 32% sucrose extraction medium and then recentrifuged at 35,000 rpm for 1 h. The obtained protein bands of tissue PM were suspended in MEM and centrifuged at 35,000 rpm for 1 h. The pellet was resuspended in the MEM and stored at 4°C.

Concentrations of isolated PM protein were determined according to the method of the Bradford (1976) using bovine plasma gamma globulin (Bio-Rad, USA) as a standard.

Polyclonal antiserum production

Polyclonal antiserum production was performed as described by Choi et al. (1998). Adult male sheep (Corriedale, 50 kg) was immunized subcutaneously on the back of neck and both sides of the groin with the PM proteins isolated from pig APM. Immunogen was suspended in saline, and mixed with Freund's complete adjuvant (F5056, Sigma Chemical) and Freund's incomplete adjuvant (F5881, Sigma Chemical). The subsequent injections of the immunogen with Freund's incomplete adjuvant were repeated twice at intervals of 3 wk following the initial immunization. Blood was collected from jugular vein of sheep prior to immunization to be used as a non-immunized serum and 12 days after the third injection as a antiserum. The blood samples were allowed to clot for 1 h at room temperature and sera were harvested by centrifugation at 3,500 rpm for 10 min at 4°C and stored at -20°C until they were used.

Lyophilized antiserum, crude immunoglobulin fraction, was prepared by 90% ammonium sulfate precipitation. The immunoglobulin fraction was dissolved in sterile distilled water at a concentration of 21.5 mg/ml, stored at 4°C until they were used.

Titer and cross-reactivity of antiserum

Titers and cross-reactivity for the prepared sheep anti-pig APM antiserum were determined by ELISA according to the method of Baek et al. (2000) and Jin et al. (2005). The absorbance was determined at 405 nm using microplate reader (Benchmark plus, model 680, Bio-rad).

Immunoblotting

SDS-PAGE for proteins isolated from various tissues (adipocyte, brain, heart, kidney, liver and spleen) of pig was performed according to the method of Laemmli (1970) and Men et al. (2005). For immunoblotting, the PM proteins separated by SDS-PAGE was blotted to nitrocellulose membrane (Hybond C-Pure, Amersham, USA) using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, USA) in blotting buffer (25 mM Tris-HCl, pH 8.3, 1.4% glycine, 20% methanol) at 4°C for 1.5 h at 110 V. Western immunoblotting was performed according to the method of Baek et al. (2000).

In vivo cross-reactivity of polyclonal antiserum

Ninety weanling pigs (male and female, 3 wk old, approximately 6-7 kg) were allocated randomly to three treatment groups (thirty pigs in each) and housed in a heated piggery. All groups were given *ad libitum* access to feed and water. Treatment groups consisted of control, antiserum and lyophilized antiserum group. Both antiserum were filtered by 0.45 μm millipore filter.

Pigs, aged 4 wk, were injected i.p. administration with 40 ml of antiserum and 20 ml of lyophilized antiserum. A control group received an 40 ml of saline. Pigs were weighed and feed intake measured monthly during all trial period. All pigs were slaughtered at 24 wk of age

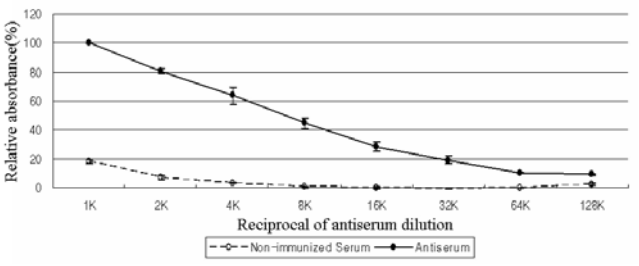

Figure 1. Reactivity of non-immunized serum and antiserum against APM proteins isolated from pig as detected by ELISA. Optical density was determined at 405 nm using microplate reader.

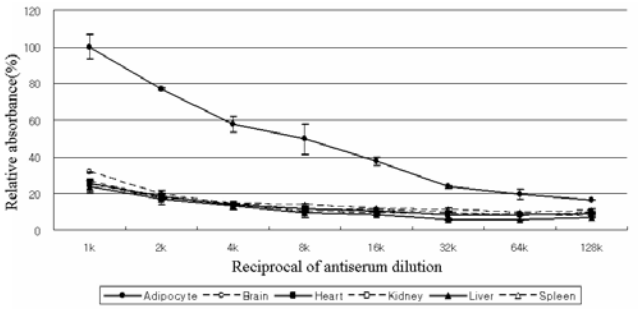

Figure 2. Cross-reactivity of antiserum with PM proteins isolated from adipocyte, brain, heart, kidney, liver and spleen of pig. Optical density was determined at 405 nm using microplate reader.

(approximately 110 kg). The carcasses were dressed according to normal commercial practice and carcass percentage, body composition and organ weight of randomly selected pigs (6 pigs/group) were recorded.

Comparisons (BW, ADG, feed intake, organ weight, carcass weight, meat composition and body composition of treatments) were made using the GLM (General Linear Model) procedure of SAS (2000). Significance was defined at $p<0.05$.

RESULT AND DISCUSSION

Assessment of antibody titer and specificity using EIA

Figure 1 illustrates the titers of antiserum raised in sheep against APM proteins isolated from pig determined by ELISA. Antiserum expressed strong antigen-antibody reactivity with APM proteins, and the reactivity could be detected at dilutions in excess of 1:128,000. Non-immunized serum taken from same sheep prior to immunization, however, had almost no reactivity with immobilized pig APM proteins. Kestin et al. (1993) also reported a high reactivity of antiserum against pig APM proteins compared to non-immunized serum. The antiserum with the highest titers was used for further studies.

Figure 2 demonstrates a cross-reactivity of antiserum against PM proteins with adipocyte, brain, heart, kidney, liver and spleen. The antiserum showed almost absolute

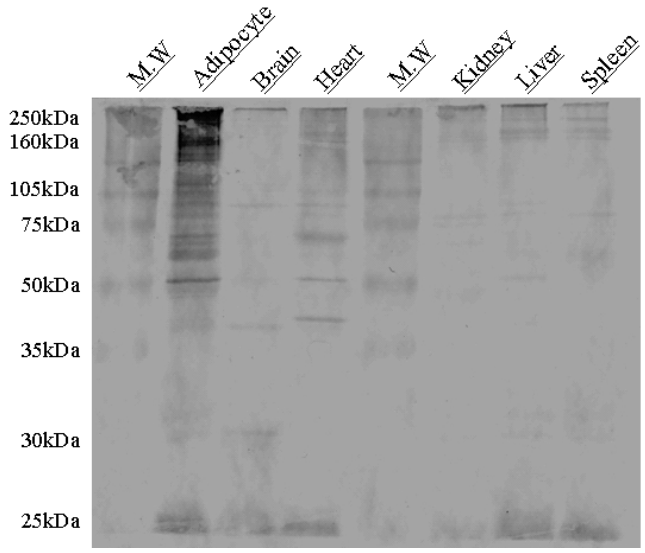

Figure 3. Western immunoblotting of various pig organ PM proteins with antiserum raised in sheep against pig APM proteins. Equivalent amount (10 μg) of membrane proteins isolated from adipocyte, brain, heart, kidney, liver and spleen were loaded at each lane. Donkey anti-sheep IgG-alkaline phosphatase (Sigma, USA) was used as a secondary antibody.

reactivity with APM proteins. At dilution of 1:1,000, however, antiserum expressed 32% reactivity with brain which was the highest among the other tissues. The other tissues maintained quite low level of reactivity with antiserum through all dilution rates. Nassar and Hu (1991a) reported that polyclonal antibodies developed against ovine APM reacted highly to APM and showed a lower level of reactivity with PM of both liver and erythrocyte. This indicates that an antiserum raised in sheep against pig APM proteins expressed adipocyte specific reactivity.

Western blotting

Figure 3 shows Western immunoblotting using antiserum against pig APM proteins as a primary antibody and donkey anti-sheep IgG-alkaline phosphatase conjugate as a secondary antibody. Although not entirely specific, the antiserum demonstrated remarkably higher degree of reactivity with APM comparing to the other tissues. Kestin et al. (1993) reported that the antiserum against pig adipocyte showed adipocyte specific reaction in immunoblot compared to the other tissues, spleen, erythrocyte, brain, liver, kidney and muscle. This is a similar finding to that of the EIA indicating a relatively high titer to adipocyte specific antigens.

In vivo cross-reactivity of polyclonal antiserum

The effects of polyclonal antiserum against pig APM protein on growth performances and feed intake are showed in Table 1. The polyclonal antiserum did not significantly change BW. ADG was not affected by the antiserum

Table 1. Effect of antiserum treatment on performance of pigs

	Control	Antiserum	Lyophilized antiserum
Initial body weight (kg)	6.4±1.03	6.6±0.94	6.2±0.69
Final body weight (kg)	103.2±4.19	105.0±5.45	105.4±5.28
Average daily gain (g)	694.03±32.98	705.67±38.23	709.48±38.56
Feed intake (kg/day/head)	1.82±0.08	1.81±0.09	1.79±0.06
Feed conversion	2.62±0.11	2.57±0.18	2.54±0.16

Table 2. Effects of antiserum against pig APM proteins raised from sheep on major organ weight of pigs (unit: kg (final weight %))

	Control	Antiserum	Lyophilized antiserum
Heart	0.39±0.04	0.39±0.06	0.42±0.04
	(0.37±0.05)	(0.35±0.05)	(0.39±0.04)
Kidney	0.33±0.02	0.32±0.02	0.31±0.02
	(0.31±0.02)	(0.29±0.01)	(0.29±0.02)
Liver	1.43±0.07	1.55±0.13	1.56±0.06
	(1.36±0.07)	(1.42±0.12)	(1.46±0.07)
Lung	0.90±0.15	0.79±0.12	0.8±0.11
	(0.86±0.16)	(0.72±0.11)	(0.75±0.10)
Spleen	0.17±0.01[a]	0.22±0.02[b]	0.19±0.03[a]
	(0.16±0.01)	(0.20±0.02)	(0.17±0.03)

[a, b] Values with different superscripts significantly differ at $p<0.05$.

Table 3. Effects of antiserum on carcass percentage of pigs

	Control	Antiserum	Lyophilized antiserum
Final weight (kg)	106.6±1.77	109.3±2.36	107.0±4.46
Carcass weight (kg)	78.7±2.25[ab]	81.4±2.70[a]	77.2±2.56[b]
Carcass percentage (%)	73.81±1.69[ab]	74.47±1.88[a]	72.14±1.04[b]

[a, b] Values with different superscripts significantly differ at $p<0.05$.

treatments in spite of numerical differences between the treatments (694.03, 705.67 and 709.48 g for control, antiserum and lyophilized antiserum, respectively). Inconsistent with the present result, Nassar and Hu (1991b) reported that polyclonal antiserum treatments to crossbred wether lambs significantly ($p<0.01$) reduced ADG.

Weights of organs (heart, kidney, liver, lung and spleen) of randomly selected pigs after slaughter are shown in Table 2. The antiserum did not affect weight of organs except for spleen. Despite lack of significance, liver was numerically heavier for antiserum treatments than for control. Present result was consistent with the previous study (Moloney et al., 2002) in which polyclonal antibodies against APM protein tended to be increase liver weight of sheep. No differences in weights of kidney and lung between the groups appearing in the present study, which is consistent with the previous results using sheep (Nassar and Hu, 1991b). Spleen enlargement could be a sign of an immune response triggered by antiserum injection (Nassar and Hu, 1991b). As expected, in the present study, spleen of pigs treated with antiserum was bigger ($p<0.05$) than for control due to activation of immunizing response by antiserum treatment. Consistent with the present results, Hu et al. (1992) reported that spleens of polyclonal antiserum against intact adipocytes treated rats tended to be larger and rather grey compared to normal spleens. However, spleen of pigs treated with lyophilized antiserum did not differ compared with control in the present study. There have been no study that compares lyophilized antiserum and antiserum in the effect of antibodies on organs and we do not know exact reason behind this phenomena. More detailed studies are necessary to elucidate the effect of immunization of lyophilized antibodies against adipocyte plasma membrane proteins.

Carcass percentage of pigs was not affected ($p>0.05$) by both antiserum treatments (Table 3). This is consistent with the previous study (Kestin et al., 1993) in which carcass percentage (mean 76.5%) was not affected by antiserum injection to subcutaneous fat of pigs. Despite lack of significances, the antiserum injection numerically increased carcass percentage compared with control. This is well associated with numerical increase of meat production by the antiserum treatment (Table 4). In addition, carcass percentage for the antiserum treated groups was significantly ($p<0.05$) higher than that for the lyophilized antiserum.

Among treatment groups, lower weight of loin and neck appeared when pigs were treated with lyophilized antiserum (Table 4). This may be partially associated with low carcass weight and percentage of lyophilized antiserum treated pigs (Table 3). Except for loin and neck, both antiserum did not affect ($p>0.05$) most of body and meat compositions. In

Table 4. Effects of antiserum on body and meat composition of pigs (unit: kg (carcass weight %))

	Control	Antiserum	Lyophilized antiserum
Body composition			
Meat	53.27±2.54	54.35±2.06	51.96±1.15
	(67.70±1.75)	(66.77±1.31)	(67.36±1.24)
Fat	6.42±0.88	6.62±0.82	6.55±1.58
	(16.36±2.41)	(16.84±2.34)	(16.91±3.72)
Bone	4.03±0.20	4.04±0.22	3.95±0.15
	(10.26±0.61)	(10.25±0.68)	(10.24±0.53)
Meat composition			
Tender loin	1.23±0.12	1.25±0.13	1.18±0.09
	(1.57±0.14)	(1.53±0.16)	(1.53±0.13)
Loin	5.52±0.57[a]	5.52±0.34[a]	4.98±0.25[b]
	(7.01±0.67)	(6.78±0.28)	(6.46±0.50)
Neck	5.72±0.39[ab]	5.93±0.41[a]	5.53±0.19[b]
	(7.27±0.44)	(7.28±0.38)	(7.17±0.34)
Belly	12.03±0.83	12.06±0.66	11.78±1.18
	(15.29±0.78)	(14.82±0.65)	(15.25±1.15)
Chuck	6.94±0.49	7.09±0.21	6.84±0.24
	(8.82±0.47)	(8.71±0.39)	(8.88±0.49)
Spare rib	2.82±0.27	3.10±0.27	2.78±0.25
	(3.59±0.33)	(3.80±0.24)	(3.61±0.41)
Hind leg	13.54±0.83	13.61±0.54	13.37±0.71
	(17.21±0.91)	(16.72±0.66)	(17.33±0.91)
Fore shank	2.42±0.26	2.54±0.26	2.45±0.16
	(3.08±0.26)	(3.12±0.32)	(3.18±0.19)
Hind shank	2.51±0.16	2.75±0.28	2.61±0.21
	(3.20±0.22)	(3.37±0.25)	(3.38±0.23)
Thin skirt	0.52±0.06	0.51±0.06	0.45±0.07
	(0.66±0.07)	(0.62±0.07)	(0.58±0.09)

[a, b] Values with different superscripts significantly differ at $p<0.05$.

particular, fat composition did not differ ($p>0.05$) between the groups. This is consistent with the previous study using chicken (Butterwith et al., 1989). However, most of studies in terms of fat-reducing antibody showed that polyclonal antiserum against APM protein reduced 20 to 30% of mesentery and subcutaneous fats (Flint, 1998; Baek et al., 2002). This discrepancy is unclear because little studies are available in relation to pigs, but it may be partially explained by different experimental environments between laboratories. For example animal dissection and isolation of fat after slaughter could be subjective to workers in a slaughterhouse. Thus, further studies taking environmental factors into account should be required.

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