RESEARCH REPORT
HUMAN NUTRITION

Title: Comparison of red meat versus high carbohydrate diet as a means of preventing tissue-specific down-regulation of insulin receptors - **NPB #13-177**

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Industry Summary:

The effect of diet on physiological and chemical balance was assessed when mature, obese gilts were fed either a high fat ground beef (GB) or a high carbohydrate corn/soybean diet (CON). A pool of 21 crossbred gilts was provided *ad libitum* access to a low lysine diet to promote over-eating and adiposity. The first 10 gilts to reach 3 cm of back fat (BF) were randomly assigned to either the GB or CON diet. Five gilts were assigned to the GB diet, which consisted of fully cooked 65:35 lean:fat ground beef top dressed with calcium carbonate (0.1%). Five gilts were also assigned to the CON diet which was made up of 70.6% ground corn, 15% vegetable oil, 8.5% dried distillers grains with solubles (DDGS) and 4.25% soybean meal. Body weight and blood draws were done on day 0, 28, 56 and 84. Back fat thickness and loin muscle area were determined via ultrasound every 28 days. Blood analysis and blood lipid panel was conducted. Gilts were humanely euthanized on day 85 for tissue collections and body composition analysis. There were no significant differences between treatments seen when using qPCR analysis for expression of IR in the longissimus, gracilis, 10^{th} rib backfat, or liver ($P = 0.43$, 0.2, 0.13, and 0.19, respectively). Tagged (immunofluorescence) insulin receptor (IR) density was not different across treatment for muscle of posture (longissimus thoracis) or subcutaneous fat collected adjacent the first thoracic vertebra. However, fluoresced IR density of GB gracilis muscle was superior to CON ($P = 0.04$) suggesting greater insulin sensitivity for this muscle of locomotion. More research is necessary involving the physiological response to food or combinations of food to determine if the obesity trend can be attributed to the increase in carbohydrate consumption and if red meat can play a role in reversing tissue-specific down-regulation of insulin receptors.

Keywords: gilts, red meat, insulin receptors, qPCR

Scientific Abstract:

Objectives: The objective of this proposal was to accurately quantify insulin receptor concentration in muscle and adipose tissue through the use of quantitative polymerase chain reaction (qPCR) techniques. .

Materials and Methods: Twenty-one gilts (Yorkshire × Duroc × Hampshire) born over a five-day period were provided *ad libitum* access to a low lysine diet (Lys = 0.45%) to promote hyperphagia and adiposity. Gilts were assigned to either a ground beef (**GB**; n = 5) or control (**CON**; n = 5) treatment upon reaching 3 cm subcutaneous backfat (**10BF**; 10/11th rib interface) and were fed for 84 d. The GB diet was 99.9% cooked ground beef (65:35 lean:fat) plus 0.1% calcium carbonate while CON comprised 70.55% ground corn, 15% vegetable oil, 8.5% DDGS and 4.25% soybean meal. Both rations met NRC requirements for gilts of this size and weight. Feed intake and orts were recorded daily. Body weights (**BW**) and blood samples were collected on d 0, 28, 56, and 84 for blood chemistry analysis. One gilt was removed from the GB treatment after d56 due to foot infection. Gilts were humanely euthanized on d 85 for tissue collections and body composition analysis. Samples of *Longissimus* thoracis muscle (**LT**; 10/11th rib interface), gracilis muscle (**GR**), 10BF, and liver tissues were snap frozen for IR qPCR analysis, and fixed in formalin for immunohistochemical evaluation of IR density.

Results: No differences were observed for mRNA expression of IR in the LT, GR, 10BF, and liver (P = 0.43, 0.2, 0.13, and 0.19, respectively). Image analyses of photomicrographs of tissues stained for IR did not differ between treatments for IR density in 10BF or pooled muscle, however GB GR IR density was significantly greater (P = 0.04) than CON GR, CON LT, and GB LT.

Conclusion: The higher density of insulin receptors in GR from ground beef-fed gilts could suggest the initiation of tissue-specific insulin resistance of that tissue. Further research is necessary to determine if consumption of a high calorie, high glycemic diet could lead to tissue-specific insulin resistance and to determine the specific metabolic role of the liver.

Introduction:

The human body deals with high blood sugar by releasing insulin produced in the beta cells of the pancreas. Insulin binds to its insulin receptors on the liver, muscle, and fat cells to serve as a key to unlock the cells ability to store glucose inside the cell. Chronic elevation of insulin ultimately leads to down-regulation of these insulin receptors. The tissue is effectively saying "*I'm full, thank you*" and over time, the hormone has less and less of an effect. This is the case of chronic insulinism. In response to elevated levels of circulating glucose, insulin does its job by replenishing energy stores of glucose (glycogen) in the liver and muscle, and triglycerides in fat (adipose) tissue. Liver and muscle are the most metabolically active of the tissues, so when the liver and muscle glucose stores remain chronically overloaded ("full"), the insulin receptors down-regulate (a mechanistic response to glycogen/glucose saturation) leaving the adipose tissue as the only remaining viable cells accepting glucose, which is converted to tri-glycerides by the liver, and stored as fat. It is this tissue-specific down-regulation of insulin receptors that leads to obesity and obesity-related metabolic disorders

Pigs are omnivores, just like humans and their anatomy and physiology are very similar to humans. A pig's gastrointestinal system, body composition, and nutrient requirements favor the use of the pig as an ideal model for evaluation of how diet influences physiological responses in growth and development (Tumbleson, 1986; Tumbleson and Schook, 1996). The National Institute of Health has stated that the pig is an excellent biomedical model with regard to the influence of diet on insulin regulation and function. Also, use of pigs as a model for humans allows the researcher to limit variation attributable to environment and genetics. Pigs can be selected from a common genetic line, a common sire, housed in a common environment, and even have a common birthdate. All of these sources of variation confound the human research and require a very large sample size.

The request for proposals called for research that is “unique or novel” and this project definitely qualifies under that classification. Tissue specific quantification of insulin sensitivity has not been developed through the means of analysis we propose; not in animals or in humans. Human studies utilizing the euglycemic clamp technique evaluate a systemic (whole body) response and cannot discern differences in quantity of insulin receptors on different tissues; specifically muscle and adipose.

Our hypothesis is that a low-glycemic, nutrient dense, red meat based diet reverses tissue specific down-regulation of insulin receptors in muscle cells with no adverse effects on cardiac health. If the hypothesis is proven true in this swine model, this could eventually lead to changes in dietary recommendations currently prescribed by health officials and physicians with regard to the ability of red meat to reverse the negative impact that refined carbohydrate-based diets have on obesity and obesity-related disorders in the United States. Ultimately this would lead to increased retail sales and **increased consumption of pork**.

Objectives:

The objective of this proposal was to accurately quantify insulin receptor concentration in muscle and adipose tissue through the use of quantitative polymerase chain reaction (qPCR) techniques.

Funding from the National Pork Board was obtained for qPCR analysis. The broader project was funded by the North Dakota Beef Commission and the North Dakota State Board of Agriculture Research and Education.

Materials & Methods: This section should include experimental design, methods and procedures used, number of animals, etc.

This study was conducted at the North Dakota State University Animal Nutrition and Physiology Center (ANPC). All animal care and handling procedures were approved by the Institutional Animal Care and Use Committee (IACUC).

Animals and Diets.

A pool of 21 crossbred gilts (Yorkshire × Duroc × Hampshire), approximately 90 days of age were selected from the NDSU swine herd and transported 0.8 kilometers to the ANPC (Fargo, ND). All pigs were born over a five-day period and had a common sire. Prior to being put on treatment, gilts were housed in a common, thermo-neutral room in individual pens (1.22 x 2.44 m). All gilts were allowed *ad libitum* access to water and a common diet formulated using the guidelines of the National Research Council dietary recommendations for growing swine (NRC, 1998) with one exception; the common diet was purposefully low in lysine to promote hyperphagia and increased adiposity. The choice of the low lysine diet was based on the findings of several studies (Witte et al., 2000; Cisneros et al., 1996). The diet was analyzed for DM, ash, CP, fat, and percent lysine (Table 1). Back fat (BF) thickness and loin muscle area (LMA) at the 10th thoracic vertebra were determined using an Aloka 500-SSD (Aloka America, Wallingford, CT). Gilts were then ultrasounded every 14 d for the remainder of the study.

The first 10 gilts to reach 3 cm of BF were selected from the pool of 21 for inclusion in the feeding trial. Gilts were put on trial as a paired sample in order to ensure multiple pigs were slaughtered on the same day and could also be compared across treatments. Upon reaching 3 cm of 10th rib backfat, gilts were randomly assigned to one of two dietary treatments stratified across litter,

BW, and BF. The control gilts (CON; n = 5) received a standard commercial grower diet formulated to NRC (1998) recommendations (Table 1). This diet consisted mainly of corn, soybean meal, and dried distillers grains (DDGS) with an additional 15% fat added in the form of corn oil. The treatment diet (GB; n = 5) was fully cooked (60:40 lean:fat) ground beef (GB) top dressed with calcium carbonate (0.10% as fed; Table 1) to meet NRC requirements for a complete balanced diet. This diet was fed four times per day at 0800, 1000, 1400 and 1600h to avoid spoilage of this high fat diet. Although it was not provided as what would be traditionally considered *ad libitum*, the GB pigs were allowed to consume as much as they wanted over these four feedings each day. The gilts on the *ad libitum* CON diet were provided 4.54 kg at 0800. If the CON rations were low at 1600 h, gilts were provided an additional 2.27 kgs to provide sufficient opportunity for *ad libitum* intake until the following morning. Orts were collected and weighed for both treatments prior to the 0800 feeding each day.

Table 1. Ingredient composition and analyzed nutrient composition of experimental diets fed to gilts for 84 days.

		Treatments	
Item	**Fattening Diet[1]**	**CON**	**GB**
Ingredient, % as fed			
Ground Beef	-	-	99.9
Corn	83.05	70.59	-
Corn oil	-	15.00	
DDGS	10.00	8.50	-
Soybean Meal	5.00	4.25	-
Di-calcium phosphate	0.345	0.29	-
Calcium	0.995	0.85	0.1
Salt	0.45	0.38	-
Swine vitamin premix[2]	0.03	0.03	-
Swine mineral premix[3]	0.14	0.12	-
Proximate Analysis			
Dry Matter	89.30	90.53	51.03
Crude Protein	9.68	11.09	16.84
Lysine	0.45	0.54	1.78
Crude Fat	4.39	16.54	36.9
Ash	3.79	3.32	0.69
Acid Detergent Fiber	5.39	7.4	-
Neutral Detergent Fiber	18.74	17.3	-

[1]Low lysine diet

[2]Vitamin premix content: vitamin A, 10,000,000 IU/lb; vitamin D3, 1,500,000 IU/lb; vitamin E, 50,000 IU/lb; vitamin B_{12}, 40 mg/lb; menadione, 4,000 mg/lb; biotin, 155 mg/lb; folic acid, 1,000 mg/lb; niacin, 50,000 mg/lb; d-panthothenic acid, 30,000 mg/lb; vitamin B_6, 3,000 mg/lb; riboflavin, 9,000 mg/lb, and thiamine, 3,000 mg/lb.

[3]Mineral premix content: Copper, 1.1%; Iodine, 240 ppm; Iron, 11.0%; Zinc, 11.0%. Manganese, 2.9%; Selenium, 200 ppm.

Ground Beef Preparation

The GB diet was prepared at the NDSU meat lab. Beef trimmings were obtained from a commercial meat processor (Long Prairie Packing Company, Long Prairie, MN). Upon arrival, trimmings were ground, spread evenly on 46 by 33 cm baking sheet pans, and cooked until done (approximately twenty-five min) at 204^{o} C. After the ground beef was cooked, it was refrigerated (3^{o} C). Allowing the product to chill ensured that the beef, fat, and juice were kept together in the pan. After the ground beef cooled for approximately 90 min, it was vacuum packaged for ease of storage. The cooked ground beef was then labeled with the date and weight, transported to ANPC, and then frozen until fed. Prior to use, ground beef packages were removed from the freezer to thaw the night before feeding. All ground beef was fed cold to maintain the consistency as the warm, soft fat appeared to be less palatable to the gilts.

Tissue Collection

Pigs were humanely euthanized after 84 days on treatment and processed under USDA Food Safety and Inspection Service guidelines. Each pig was slaughtered on day 85 after completing 84 days. Test pigs were slaughtered on three slaughter days (n = four, two, and four pigs). A modified necropsy was performed and weights were obtained for adrenal glands, heart, liver, pancreas, spleen and perirenal fat (weights reported in Wellnitz et al., 2013) as well as right and left ventricle thickness measurements (top, middle and bottom).

Samples from the Gracillus muscle (GM), Longissimus Thoracis muscle (LT), 10th rib backfat and liver tissues were collected and snap frozen for later quantitative PCR analysis. Tissues were homogenized in 30 mg sections by a polytron fitted with a 7 mm generator. After homogenization, RNA was isolated using the Qiagen RNeasy kit according to the manufacturer's instructions. Quantification of RNA samples was done using a Nanodrop 2000c spectrophotometer. Complementary DNA (cDNA) was synthesized using RNA from each sample in a reverse transcription reaction using the Qiagen QuantiTect RT Kit. As opposed to traditional PCR, Quantitative PCR (qPCR) was utilized to identify the number of insulin receptors after each PCR cycle. Gene expression was determined using the Taqman primer probe sets designed for each gene and qPCR. The probe is a florescence primer that binds to a specific part of the DNA and has what are called a reporter and quencher. Once the polymerase began to copy DNA during the PCR, the primer probe became bound and lysed (eliminated) via exonuclease activity, and subsequently the reporter was administered and florescence emitted. The qPCR method measured the amount of florescence and number of receptors was determined. Within each sample, expression of every gene was normalized to the 18s rRNA control within each time point. Two treatment qPCR plates were run for comparison. The data collected was used to quantify the number of receptors on muscle, liver, and fat tissue samples as a means to determine statistical differences between treatments.

Statistical Analysis

Data were analyzed using the mixed procedure of SAS (SAS Institute Inc., Cary, NC) and generalized least square means as repeated measures with the fixed effects of treatment, day, and treatment × day with pig ID serving as the repeated/subject variable. The ten gilts chosen based on the previously described selection criteria were a fixed effect due to the fact that they were chosen as the first 10 to reach the specified fat thickness.

Results: Report your research results by objective.

The National Pork Board funded one specific objective of a much larger research project. The objective of this NPB-funded proposal was to accurately quantify insulin receptor concentration in muscle and adipose tissue through the use of quantitative polymerase chain reaction (qPCR) techniques. The data were analyzed using the Comparative Ct method and statistics were determined using the mixed procedure of SAS (SAS Inst. Inc., Cary, NC). There were no differences between treatments seen when using qPCR analysis for expression of insulin receptors in the LT, GM, 10th rib backfat, and liver (P = 0.43, 0.2, 0.13, and 0.19, respectively).

Discussion:

Our results concluded that there were no significant differences between treatments seen when using qPCR analysis for expression of insulin receptors in the LT, GM, 10th rib backfat, and liver (P = 0.43, 0.2, 0.13, and 0.19, respectively). In other words, neither diet triggered an increase (or decrease) in genetic coding for insulin receptors in the tissues tested. No strong conclusions can be made from this single test.