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Arterial insulin resistance in Yucatan micropigs with diet-induced obesity and metabolic syndrome

Cecilia C. Low Wang¹, Li Lu², J. Wayne Leitner¹, Mohammad Sarraf², Roberto Gianani³, Boris Draznin¹, Clifford R. Greyson², Jane E. B. Reusch¹, and Gregory G. Schwartz²

¹Endocrine Section, VA Medical Center, Denver, CO and University of Colorado Anschutz Medical Campus/School of Medicine, Aurora, CO

²Cardiology Section, VA Medical Center, Denver, CO and University of Colorado Anschutz Medical Campus/School of Medicine, Aurora, CO

³Barbara Davis Center for Childhood Diabetes, Aurora, CO

Abstract

Aim—Metabolic syndrome affects a large proportion of the population and increases cardiovascular disease risk. Because metabolic syndrome often co-exists clinically with atherosclerosis, it is difficult to distinguish their respective contributions to vascular abnormalities. Accordingly, we utilized a porcine dietary model of metabolic syndrome without atherosclerosis to investigate early abnormalities of vascular function and signaling.

Methods—Thirty-two Yucatan micropigs were fed either a high fat, high simple sugar, high calorie (HFHS) or standard chow diet (STD) for 6 months. Neither diet contained added cholesterol. Blood pressure and flow-mediated vasodilatation were assessed at baseline and 6 months. Aortas were harvested at 6 months to assess histology, insulin signaling, and endothelial nitric oxide (eNOS) phosphorylation.

Results—HFHS pigs developed characteristic of metabolic syndrome including obesity, dyslipidemia, and insulin resistance, but without histological evidence of atherosclerosis. Although arterial intima-media thickness did not differ between groups, vascular dysfunction in HFHS was manifest by increased blood pressure and impaired flow-mediated vasodilation of the femoral artery. Compared with STD, aortas from HFHS exhibited increased p85 α expression and Ser307 IRS-1 phosphorylation, and blunted insulin-stimulated IRS-1-associated phosphatidylinositol (PI) 3-kinase activity. In the absence of insulin stimulation, aortic Akt Ser473-phosphorylation was greater in HFHS than in STD. With insulin stimulation, Akt phosphorylation increased in STD, but not HFHS. Insulin-induced Ser1177-phosphorylation of eNOS was decreased in HFHS, compared with STD.

Conclusions—Pigs with metabolic syndrome develop early vascular dysfunction and aortic insulin signaling abnormalities, and could be a useful model for early human vascular abnormalities in this condition.

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Address all correspondence and requests for reprints to: Dr. Cecilia C. Low Wang, MS8106, RC-1 South Room 7103, 12801 East 17th Avenue, Aurora, CO 80045, (303) 724-7801, (fax) (303) 724-3920, Cecilia.Wang@UCDenver.edu.

Conflict of Interest Statement

The authors declare that they have no conflict of interest that could bias the studies described.

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Keywords

swine; flow-mediated vasodilatation; insulin resistance; metabolic syndrome; blood pressure; endothelial nitric oxide synthase

INTRODUCTION

Metabolic syndrome affects approximately one third of adults in the United States (Ervin, 2009) and also poses an increasing problem in children and adolescents, among whom the prevalence approaches 10% (Johnson et al., 2009). Although insulin resistance is not a criterion for the diagnosis of metabolic syndrome, a substantial majority of individuals with metabolic syndrome are also insulin resistant (Eckel et al., 2005). Even in the absence of diabetes, metabolic syndrome elevates cardiovascular risk (Hu et al., 2002). This excess risk is out of proportion to atherosclerotic burden (Bayturan et al., 2010), suggesting a role for other factors that may be related to insulin resistance.

Insulin resistance may convey risk indirectly through associated metabolic abnormalities such as elevated circulating free fatty acids or hypoadiponectinemia, which disrupt vascular endothelial function and promote hypertension (Berg and Scherer, 2005; Boden, 2011). Insulin resistance may also adversely affect vascular function directly. For example, rats with genetic or diet-induced insulin resistance, but without diabetes, demonstrate impaired endothelial function (Panchal et al., 2011) and increased aortic stiffness (Sista et al., 2005), which are each associated with increased cardiovascular risk (Gokce et al., 2003; Mitchell et al., 1997). Furthermore, deletion of the insulin receptor in an endothelial cell-specific manner results in endothelial dysfunction (Rask-Madsen et al., 2010), indicating that intact insulin signaling is required for normal vascular function. Physiologic signaling by insulin in the arterial wall also promotes increased glucose utilization, physiologic growth responses, and activation of cellular pro-survival pathways, which may help to maintain normal vascular structure and function (Baron, 1996; Zeng et al., 2000).

Attenuated insulin signaling through the phosphatidylinositol (PI) 3-kinase/Akt pathway is a hallmark of insulin resistance (Cusi et al., 2000; Muniyappa et al., 2007), and may affect downstream targets of Akt such as endothelial nitric oxide synthase (eNOS) (Hsueh and Law, 1999). In contrast, insulin signaling through extracellular signal-regulated kinase (ERK) mitogen-activated protein (MAP) kinase pathways is usually preserved or enhanced in insulin resistance (Jiang et al., 1999). An imbalance between attenuated PI 3-kinase/Akt signaling and preserved MAP kinase signaling in insulin resistance may contribute to the development of endothelial dysfunction, hypertension, and atherosclerosis (Hsueh and Law, 1999; Montagnani et al., 2002; Muniyappa et al., 2007). Furthermore, tonic Akt activation by chronic insulin stimulation due to compensatory hyperinsulinemia may have deleterious effects (Bertacca et al., 2005; Robinson and Buse, 2008).

We have previously developed and characterized a porcine model of diet-induced obesity that results in insulin resistance without diabetes and recapitulates many of the features of metabolic syndrome (Lee et al., 2010). Because the intervention diet contains no added cholesterol, it does not promote atherosclerosis, providing a model that allows the effects of metabolic syndrome to be assessed independently of atherosclerosis. In this model, we have previously demonstrated that systemic insulin resistance is accompanied by the development of insulin resistance in skeletal muscle and myocardium (Lee et al., 2010). The current study determined whether porcine metabolic syndrome results in abnormal vascular function, arterial structure, and insulin signaling in the arterial wall.

MATERIALS and METHODS

The National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals was followed, and all experiments were approved by the Institutional Animal Care and Use Committee prior to being performed.

Porcine model and experimental design

Thirty-two Yucatan micropigs (16 females and 16 castrated males) were obtained at approximately 3 months of age (S&S Farms, Ramona, CA, USA). Other data from these pigs have been previously published (Huang et al., 2013; Lee et al., 2010). After a 2-week period of acclimatization to the laboratory environment with a diet of standard, low-fat minipig chow (LabDiet 5L80, St Louis, MO), pigs were sedated with ketamine 25 mg/kg IM followed by continuous infusion of ketamine 15 mg/kg/hr IV and midazolam 0.35 mg/kg/hr IV and underwent baseline measurements of weight, fasting blood glucose (YSI 2300, Yellow Springs, OH, USA), plasma insulin (Beckman Coulter Access Ultrasensitive Insulin, Fullerton, CA, USA), free fatty acids, cholesterol, and triglycerides (Wako, Richmond, VA, USA). Pigs also underwent measures of blood pressure, flow-mediated vasodilation, and intravenous glucose tolerance testing (IVGTT).

Blood pressure was measured non-invasively with an automated sphygmomanometer. Six measurements over approximately 1 hour were averaged for each pig. In a subset of 16 pigs, flow-mediated vasodilatation (FMD) of the femoral artery was assessed. A blood pressure cuff was placed around the lower leg. The femoral artery proximal to the blood pressure cuff was imaged using a 9MHz ultrasound transducer. The cuff was inflated to a pressure 50 mmHg higher than systolic blood pressure for 5 min. Cessation of flow in the femoral artery was confirmed with a handheld Doppler probe. After 5 minutes of occlusion, the cuff was released, reactive hyperemia was confirmed with the handheld Doppler probe, and the femoral artery was re-imaged for the ensuing 5 min. All images were digitized and gated to the cardiac cycle. Femoral artery internal diameter was measured by an investigator blinded to diet assignment.

All pigs underwent IVGTT as previously described (Lee et al., 2010). Briefly, glucose (0.3 g/kg) was injected intravenously over 5 min. Blood sampled from an internal jugular vein at frequent intervals over the following 100 min was obtained for measurement of blood glucose and plasma insulin concentrations. Areas under the curve for glucose and insulin were calculated.

Following these baseline assessments, pigs were assigned to receive either standard low-fat chow (STD) or chow enriched in saturated fat and simple sugars (HFHS). Equal numbers of male and female pigs were assigned to each diet. Diet compositions have been described previously (Lee et al., 2010), are specified in the Supplementary Table, and are summarized briefly as follows: the STD diet consisted of high fiber minipig chow (LabDiet 5081) without added fats or sugars fed at 30 g/kg per day, allowing for normal growth and development. The macronutrient composition of the STD diet consisted of: 9% of calories from fat (of which 11% was saturated), 71% from carbohydrates, and 20% from protein. The HFHS diet provided caloric excess in the form of minipig chow (LabDiet 5L80) supplemented with coconut oil (25% wt/wt, Cargill Ultimate 76) and high fructose corn syrup (20% wt/wt, Cargill Isoclear 55) fed at 50 g/kg per day. The macronutrient composition of the HFHS diet was as follows: 45% of calories from fat (of which 90% was saturated), 47% from carbohydrates, and 8% from protein. The two diets were similar in quantity (but not percentage) of protein, essential fatty acids, and minerals provided. Cholesterol was not included in either diet to avoid development of atherosclerosis. All baseline measurements were repeated after 6 months of assigned diet.

Several days later, pigs underwent a terminal experiment under general anesthesia. After sedation with ketamine (25 mg/kg IM) and midazolam (0.5 mg/kg IM, pigs were anesthetized with alpha-chloralose (100 mg/kg IV induction, 20–30 mg/kg/hr IV maintenance) and midazolam (0.35 mg/kg/hr IV). The chest was opened by median sternotomy to expose the heart and thoracic aorta. Measurements of cardiac function and metabolism were performed and have been reported previously (Huang et al., 2013; Lee et al., 2010). In 16 pigs (8 from each diet group), euthanasia and harvesting of the thoracic aorta were performed without administration of exogenous insulin. In 16 other pigs (8 from each diet group), a saturating bolus dose of insulin (10 U/kg IV) was administered 10 minutes prior to euthanasia and harvesting of the thoracic aorta. The latter group had undergone an antecedent euglycemic hyperinsulinemic clamp, results previously reported (Lee et al., 2010). There was an intervening period of at least 2, and up to 4 hours between the end of the clamp and the administration of the saturating bolus dose of insulin. This intervening period was sufficient to restore responsiveness of PI3K/Akt signaling to insulin (Lee et al., 2010; Traxinger and Marshall, 1989). After euthanasia, the thoracic aorta was harvested in all pigs.

Preparation of aortic lysates

Frozen full-thickness samples of aorta were pulverized, mixed with mammalian lysis buffer (150 mmol/l sodium chloride, 1 mmol/l ethylenediaminetetraacetic acid (EDTA), 1 mmol/l ethylene glycol tetraacetic acid (EGTA), 5 mmol/l sodium pyrophosphate tetrabasic decahydrate, 1 mmol/l sodium orthovanadate, 20 mmol/l sodium fluoride, and 500 mmol/l okadaic acid in dimethyl sulfoxide), homogenized with a Polytron homogenizer 5 times at 4 seconds, and centrifuged. Supernatants were assayed for protein content using a bicinchoninic acid reagent (Pierce Biotechnology, Rockford, IL, USA). Lysates were stored at –80°C until used.

Insulin receptor substrate (IRS)-1-associated PI 3-kinase activity

As previously described (Pereira et al., 2008), aliquots of aortic lysates were pre-incubated with IRS-1 antibody in a 1:100 dilution (Millipore, Billerica, MA, USA). Protein A/G agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA, USA) pre-coupled with rabbit IgG were added and rotated overnight. After centrifugation, beads were washed thoroughly, and pelleted.

Samples were resuspended in PI 3-kinase buffer (25 mmol/l 3-(n-morpholino)propanesulfonic acid, 1 mmol/l EGTA, and 5 mmol/l magnesium chloride in distilled water, titrated to pH 7.0) and PI 3-kinase reaction mixture which included sonicated L-alpha-phosphatidylinositol and 5-fold concentrated PI 3-kinase buffer. The reaction was allowed to proceed for 30 min after adding gamma-³²P-phosphate-ATP. The organic layer was extracted twice with chloroform, combined and dried. Samples were resuspended in a 2:1 chloroform:methanol solution just before spotting on thin-layer chromatography plates which had been dried after activating with 1 mmol/l EDTA, 1% potassium oxalate, and 40% methanol. Plates were placed in developing solution consisting of 65% 1-propanol and 1% glacial acetic acid, then left overnight. Plates were then air-dried and exposed to film. Densitometry of the thin-layer chromatography spots was performed using a Bio-Rad Fluor-S MultiImager, and quantified using QuantOne software (Bio-Rad, Hercules, CA, USA).

Western blotting

Aliquots of aortic lysates were suspended in Laemmle sample buffer, and stored at –20°C until used. Samples were boiled for 10 min, and equal amounts of protein were loaded in each lane, and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) before transfer to polyvinylidene fluoride membranes or nitrocellulose membranes.

Membranes were probed with primary antibodies for Akt, Ser473 phosphorylated Akt, ERK 1/2, phosphorylated ERK 1/2, eNOS, or Ser1177 phosphorylated eNOS (all from Cell Signaling Technology, Danvers, MA, USA), or p85alpha (Millipore, Billerica, MA, USA), at 1:1000 dilution. For detection of Ser307 phosphorylated IRS-1, aliquots of aortic lysates were immunoprecipitated for IRS-1 as described above, then probed with primary antibody for Ser307 phospho-IRS-1 (Millipore, Billerica, MA, USA). Each membrane was incubated with appropriate secondary antibody conjugated with horseradish peroxidase. Protein content was detected by ECL kit (GE Biosciences, Picataway, NJ, USA). Densitometry was performed as described above.

Detection of Akt isoforms

Total content of Akt1, Akt2, and Akt3 isoforms (phosphorylated + non-phosphorylated forms) in aorta was determined by Western blotting using isoform-specific antibodies (Cell Signaling Technology, Danvers, MA, USA) at dilutions of 1:1000. To measure the phosphorylated forms of each Akt isoform, aliquots of aortic lysates underwent immunoprecipitation for Akt1, Akt2 or Akt3 using the specific antibodies at dilutions of 1:500, 1:50 or 1:100, respectively. After SDS-PAGE and transfer to polyvinylidene fluoride membranes, immunoblotting was performed with isoform-nonspecific antibody for Ser473 phosphorylated Akt. Appropriate secondary antibody conjugated with horseradish peroxidase was used. Protein content was measured and densitometry performed as described above.

Aortic histology

Formalin-fixed aortic sections were embedded in paraffin, sectioned at 7 micron intervals, stained with hematoxylin and eosin, and photographed with an Olympus microscope connected to a Pixera digital camera (Pixera Corporation, San Jose, CA, USA). Sections were examined for fat deposition or nascent atherosclerotic lesions. Morphometric analysis of digitized images was performed with Image Pro Plus software (Media Cybernetics, Inc., Bethesda, MD, USA) by a pathologist blinded to diet assignment. Aortic sections were also stained for elastin using the Van Gieson elastin stain, and intima-medial thickness measured by a microscopist blinded to diet assignment.

Statistical Analyses

Results are expressed as mean $\pm$ SE. Two-way ANOVA was used to determine interactions between diet and time, or diet and exogenous insulin dose in vivo. Unpaired Student's *t* test was used to compare diet groups at each time point, or in absence or presence of insulin. Paired Student's *t* test was used to compare data within diet assignment between baseline and end-of-study time points. A *p*-value of <0.05 was considered to be of statistical significance. No differences were observed between male and female pigs, but this study was not powered to detect gender differences within diet assignment and insulin treatment. Therefore, data for both genders were combined for analyses.

RESULTS

Pigs fed intervention diet developed features recapitulating clinical metabolic syndrome without diabetes

Compared with the STD group, pigs fed HFHS diet developed obesity, dyslipidemia, mildly elevated fasting blood glucose and insulin, and elevated systolic blood pressure (Fig. 1A, Table 1). In addition, after 6 months of assigned diet, pulse pressure was 56 ± 5 mmHg in HFHS, compared with 46 ± 3 mmHg in STD ($p=0.08$). Baseline IVGTT showed similar areas under the curve for glucose and insulin in both diet groups. However, at 6 months of

assigned diet, the areas under the curve for both glucose and insulin rose to significantly higher levels in the HFHS group, compared with STD (Table 1).

Flow-mediated vasodilation (FMD)

Femoral artery FMD was similar at baseline in both groups, and did not change significantly over time in the STD group. However, FMD decreased significantly after 6 months of HFHS diet (Fig. 1B).

Histologic examination and intima-media thickness

The thoracic aortas did not demonstrate fatty streaks or more advanced atherosclerotic lesions by gross inspection or histologic examination. Intima-media thickness did not differ between diet groups (Fig 1C).

Insulin-stimulated, insulin receptor substrate (IRS)-1-associated PI3-kinase activity

In the absence of acute insulin administration *in vivo*, insulin receptor substrate (IRS)-1-associated PI 3-kinase activity was similar in both diet groups (Fig. 2A). Insulin significantly increased PI3-K activity in both diet groups, but the effect of insulin was markedly blunted in HFHS compared with STD. After insulin exposure, PI 3-kinase activity was significantly greater in STD than in HFHS ($p<0.001$) with significant interaction of diet and insulin on PI 3 -kinase activity ($p<0.001$).

Aortic expression of PI 3-kinase subunit p85 α and Ser307 phosphorylation of IRS-1

In order to explore molecular mechanisms for decreased insulin mediated PI 3-kinase activity in the aorta, expression of the p85 α regulatory subunit of PI 3-kinase was examined in the aorta. Total p85 α expression was significantly higher in aortic lysates from the HFHS group than from the STD group (Fig. 2B, $p<0.05$). Phosphorylation of IRS-1 at the Ser307 residue was examined, and found to be significantly higher in aortic lysates from the HFHS group compared with the STD group ($p<0.001$, Supplemental Fig 1).

Insulin-stimulated total and isoform-specific Ser473 Akt phosphorylation

To identify downstream effects of impaired IRS-1-associated PI 3-kinase activity in aorta, we examined insulin-stimulated Ser473 phosphorylation of Akt and its major isoforms. In the STD diet group, total (isoform-nonspecific) Akt phosphorylation increased in response to insulin, as expected. In contrast, the HFHS group exhibited significantly greater Akt phosphorylation than STD in the absence of insulin, but no additional phosphorylation in response to insulin exposure (Fig. 3A). The interaction of diet and insulin on phospho-Akt was highly significant ($p<0.001$). Total (phosphorylated and non-phosphorylated) Akt did not differ between diet groups in the absence or presence of insulin stimulation.

Differences in Akt phosphorylation in STD versus HFHS diet groups were investigated further by examination of isoform-specific Akt phosphorylation. There was a trend suggesting greater Akt1 phosphorylation in STD than in HFHS ($p=0.07$ for main effect of diet). In response to insulin in STD, Akt1 phosphorylation was modestly, but not significantly increased. No effect of insulin stimulation on phospho-Akt1 was apparent in HFHS (Fig. 3B). There was a trend suggesting Akt2 phosphorylation in response to insulin in STD ($p=0.07$, Fig. 3C). Akt3 phosphorylation differed significantly between diet groups and demonstrated a pattern qualitatively similar to total phospho-Akt: In STD, phospho-Akt3 increased significantly with insulin treatment ($p=0.03$). Phospho-Akt3 was unchanged between baseline and with insulin in the HFHS group (Fig. 3D). The interaction of diet and insulin on phospho-Akt3 was significant ($p<0.05$). The total (phosphorylated and non-

phosphorylated) content of each Akt isoform did not differ significantly between diet groups, either in the absence or presence of insulin treatment.

Endothelial nitric oxide synthase (eNOS) phosphorylation (Ser1177)

Insulin ordinarily promotes Ser1177 phosphorylation of eNOS via PI 3-kinase/Akt (Montagnani et al., 2001; Zeng et al., 2000). In the absence of insulin treatment, phospho-eNOS was similar in both diet groups. After exposure to insulin, phospho-eNOS was significantly greater in STD than in HFHS ($p < 0.01$, Fig. 4). In STD, the comparison of phospho-eNOS with and without insulin suggested an increase with insulin ($p = 0.07$), but no such difference was apparent in the analogous comparison in HFHS. The interaction of diet and insulin treatment on phospho-eNOS was significant ($p < 0.05$). Total eNOS did not differ between diet groups in the absence or presence of insulin treatment *in vivo*.

ERK 1/2 MAPK phosphorylation increased on intervention diet

ERK 1/2 MAP kinase is a major MAP kinase activated by insulin. In the absence of insulin, phospho-ERK 1/2 MAP kinase was greater in HFHS than in STD ($p < 0.05$). With insulin stimulation, phospho-ERK 1/2 MAP kinase increased significantly in STD, but no further increase was observed in HFHS (Supplemental Fig 2).

DISCUSSION

In a porcine dietary model that recapitulates the features of metabolic syndrome, the current study demonstrates that the development of systemic insulin resistance is accompanied by vascular dysfunction (hypertension and impaired FMD) and abnormal insulin signaling in the arterial wall. The latter encompasses attenuated insulin-stimulated activation of PI 3-kinase, elevated PI 3-kinase subunit p85 α expression, high baseline Akt phosphorylation at Ser473 without further response to insulin, loss of eNOS Ser1177 phosphorylation in response to insulin, and increased signaling through ERK 1/2 MAP kinase. These abnormalities develop in the absence of diabetes or atherosclerosis. This realistic, large animal model may be relevant for further understanding of the longitudinal development of human vascular dysfunction in metabolic syndrome.

Other investigators have induced insulin resistance in porcine models utilizing atherogenic diets enriched with cholesterol and fat, along with islet cell destruction (Dyson et al., 2006; Gerrity et al., 2001). Although these models have provided valuable information, the development of overt atherosclerosis and marked hyperglycemia in conjunction with insulin resistance precludes the ability to distinguish their independent effects on the arterial wall. Porcine models for metabolic syndrome have recently been developed (Kreutz et al., 2011; Robich et al., 2011) but studies of the vascular wall in these models have not been published. The present experimental model affords three distinct advantages. First, the intervention diet, enriched in saturated fat and simple sugars but not cholesterol, allows abnormalities of arterial function and signaling to be discerned without confounding by concomitant atherosclerosis or hyperglycemia. Second, the present dietary porcine model has broad clinical relevance, recapitulating features observed in many patients with metabolic syndrome. Third, as gauged by levels of fasting glucose and insulin, the degree of systemic insulin resistance in the present model is mild. This has considerable clinical relevance, because humans experience a gradual progression of insulin resistance, most often without significant hyperglycemia at the early stages. At the same time, abnormalities in arterial function are observed clinically early in the course of human insulin resistance (Duncan et al., 2007).

By the time atherosclerosis is detected in patients, thickening of the intima and the media is present and predicts subsequent cardiovascular events and mortality (Westerbacka et al., 2005; Wofford et al., 1991). Likewise in pig models of insulin resistance complicated by diabetes and/or atherosclerosis, extensive pathologic arterial remodeling is observed, including increased intima-medial thickness. However, in the present porcine model of insulin resistance without diabetes or atherosclerosis, intima-media thickening was not observed despite defects in insulin signaling, blood pressure and endothelial function. This suggests that the insulin signaling defects identified in the present model occur early in the development of insulin-resistant arteriopathy, antecedent to gross structural abnormalities of the arterial wall. These insulin signaling defects are observed in conjunction with abnormal vasomotion in the form of elevated blood pressure and defective flow-mediated endothelium-dependent vasodilatation.

Defects in insulin signaling through PI 3-kinase/Akt are a hallmark of insulin resistance and may contribute to pathologic changes in the arterial wall (Muniyappa et al., 2007). Proximal in the signaling cascade, we found that insulin-stimulated PI 3-kinase activation was blunted in aortas from pigs fed HFHS diet, compared with those from pigs fed STD diet. Excess p85 α expression can cause non-functional p85 α monomers to compete with functional p85-p110 heterodimers for IRS-1 binding sites, thus limiting the ability of insulin stimulation to augment PI 3-kinase activity (Barbour et al., 2005). Alternatively, PKC activation in endothelial cells may interfere with PI 3-kinase signaling through phosphorylation of Thr86 of p85/PI 3-kinase as outlined in a recent report (Maeno et al., 2012). We have previously demonstrated that the mechanism for insulin resistance in myocardium of the current pig model involves increased phosphorylation of IRS-1 at Ser307 (Lee et al., 2010). Our current findings suggest that in addition to increased Ser307 phosphorylation of IRS-1, excess p85 α subunit expression may also contribute to vascular insulin resistance in this model of the metabolic syndrome.

The mechanisms for vascular abnormalities in the setting of insulin resistance and diabetes are complex and not well-understood. Genetic models support the notion that impaired insulin signaling through PI 3-kinase/Akt leads to vascular dysfunction and promotes the development of atherosclerosis. For example, mice with endothelial deletion of the insulin receptor and knockout of apolipoprotein E (EIRAKO mice) fed a high fat diet demonstrated decreased Akt activation by insulin in association with impaired endothelium-depending vasodilatation and accelerated atherosclerosis (Galkina et al., 2012). In that model however, PI 3-kinase activity and p85 α expression were not characterized. Insulin receptor/IRS-1 double heterozygous apolipoprotein E knockout mice also demonstrate excess atherosclerosis (Galkina et al., 2012). Mice heterozygous for germline deletion of the insulin receptor (IRKO) demonstrate only mild systemic insulin resistance, but develop elevated blood pressure in association with decreased basal nitric oxide bioactivity and insulin-mediated phosphorylation of eNOS (Wheatcroft et al., 2004). In contrast, both EIRAKO mice and mice with endothelial-specific deletion of the insulin receptor alone with intact apolipoprotein E expression (VENIRKO mice) exhibit normal or lower blood pressure than wild-type mice (Vicent et al., 2003). Mice with germline deletion of the insulin receptor but re-expression in the brain, liver and pancreatic β -cells (TTr-IR^{-/-}) also exhibit normal blood pressure and vascular function (Symons et al., 2009). This demonstrates the difficulty of using these models to prove or disprove the significance of insulin's effects on vascular tone since deletion of the insulin receptor eliminates both insulin stimulation of eNOS phosphorylation via the PI 3-kinase/Akt pathway as well as endothelin-1 expression via the ERK MAP kinase pathway.

The current study shows that pigs fed HFHS diet also demonstrated abnormalities of Akt activation in the arterial wall. Pigs in the STD group demonstrated the expected, robust

phosphorylation of Akt in response to insulin *in vivo*. In contrast, pigs in the HFHS group had high levels of Akt phosphorylation in the absence of exogenous insulin, and no further increase in Akt phosphorylation in response to exogenous insulin *in vivo* (Fig. 3). These findings are consistent with prior studies in other models demonstrating a differential effect of acute vs. chronic insulin stimulation on Akt activation (Bertacca et al., 2005; Robinson and Buse, 2008), and are qualitatively similar to findings in skeletal and cardiac muscle using the present model (Lee et al., 2010). A substantial body of evidence indicates that while transient activation of Akt in response to insulin or physiologic stress has protective, cell-survival effects, tonic activation of Akt may have deleterious effects (Matsui et al., 2001). Nagoshi, et al. demonstrated that a transgenic mouse model of chronic Akt activation in a cardiac-specific manner led to feedback inhibition and blunting of PI3-kinase activity, resulting in increased cardiac injury and decreased functional recovery after ischemia-reperfusion injury (Nagoshi et al., 2005).

The isoforms Akt1, Akt2 and Akt3 are products of distinct genes but are highly related, with more than 80% protein identity. There is evidence for some overlapping and compensatory functions among the three Akt isoforms (Gonzalez and McGraw, 2009). Upon immunoprecipitation and analysis of phosphorylated forms of each Akt isoform, we did not find that any single isoform fully accounted for the differences in total phospho-Akt between diet groups. This is not surprising due to the non-quantitative nature of immunoprecipitation and immunoblotting. Nonetheless, directional effects of diet on each of the Akt isoforms were consistent with the effect of diet on total phospho-Akt. For example, in the absence of insulin stimulation, phospho-Akt2 and -Akt3 tended to be greater in HFHS than in STD. Under insulin stimulation, phospho-Akt1 tended to increase and phospho-Akt3 significantly increased in STD, but not in HFHS, with significant interaction of diet and insulin on phospho-Akt3. Akt3 is expressed in substantial amounts in human aortic vascular smooth muscle cells and ordinarily undergoes robust activation by insulin (Sandrasegaran and Kester, 2001). Akt3 may control vascular endothelial growth factor (VEGF) secretion and angiogenesis (Liby et al., 2012), and is required for VEGF stimulation of mitochondrial biogenesis (Wright et al., 2008). Furthermore, blunted insulin-stimulation of Akt3 activity has been demonstrated in skeletal muscle of obese individuals (Brozinick, Jr. et al., 2003). However, the specific vascular effects of blunted Akt3 signaling in response to insulin are unknown and warrant further study.

An important mechanism for hypertension in insulin resistance is decreased phosphorylation of eNOS (Muniyappa et al., 2007). Insulin ordinarily promotes endothelial nitric oxide production and endothelium-mediated vasodilation via PI 3-kinase/Akt mediated phosphorylation of eNOS (Montagnani et al., 2001). Conversely, impaired insulin signalling in endothelium causes vascular dysfunction. Endothelium-specific deletion of the insulin receptor impairs eNOS phosphorylation and endothelium-mediated vasodilatation in apolipoprotein E knockout mice (Rask-Madsen et al., 2010). The observations of the present study are consistent with sequential defects in activation of PI 3-kinase, Akt, and eNOS by insulin which may contribute to early but pronounced vascular dysfunction in this model of metabolic syndrome.

However, other studies question the role of vascular insulin signaling in the development of hypertension and vascular dysfunction. For example, C57BL/6J mice fed a high-fat diet develop type 2 diabetes and impaired endothelium-dependent vasodilatation, but intact aortic and femoral arterial insulin-stimulated Akt and eNOS phosphorylation (Molnar et al., 2005). The mechanism for impaired vascular function in this model was identified as disruption of arterial eNOS dimer formation. Furthermore, mice with germline deletion of Akt1 (Akt1^{-/-}) do not exhibit significantly elevated blood pressure, decreased eNOS phosphorylation or impaired endothelium-dependent vasodilatation (Symons et al., 2009).

The difficulty with drawing conclusions about the importance of insulin-stimulated PI 3-kinase/Akt activation in vascular function in this latter model is that Akt2 and Akt3 may be playing compensatory roles.

In addition to activation of the PI 3-kinase/Akt cascade, insulin activates several mitogen-activated protein kinases, including ERK 1/2. Activation of ERK 1/2 can promote proliferation and migration of vascular smooth muscle cells and production of endothelin-1 by endothelial cells (Potenza et al., 2005; Wang et al., 2004). In contrast to blunted insulin signalling through the PI 3-kinase/Akt/eNOS axis, we found increased phosphorylation of ERK 1/2 MAP kinase in arterial tissue from pigs with features of the metabolic syndrome both in the absence and presence of acute insulin stimulation. This contrast mirrors that observed previously in rodent models of insulin resistance and suggests that this imbalance may also be important in clinical pathophysiology of hypertension and endothelial dysfunction in patients with insulin resistance (Cusi et al., 2000; Jiang et al., 1999; Muniyappa et al., 2007).

Several limitations of the current study are recognized. The fidelity of blood pressure measurements under moderate sedation to ambulatory blood pressure is uncertain (Goodrich et al., 2001); nonetheless, a difference in blood pressure between groups was demonstrated despite sedation. FMD may reflect both endothelium-dependent and endothelium-independent vasodilator responses; although we cannot exclude the possibility that metabolic syndrome affected endothelium-independent vasodilation in our model, there is no precedent for such an effect in experimental or clinical studies (Higashi et al., 2001; Steinberg et al., 1996). Therefore, it is reasonable to infer impaired vascular endothelial function from impaired FMD. We did not harvest femoral arteries or assess FMD in our initial cohort of insulin-treated pigs. Therefore, we do not have parallel measures of insulin signaling and vascular function in the same arteries. It is uncertain whether impaired FMD in our model reflects a direct effect on endothelial function, an effect mediated by reduced insulin signaling, or a combination of both. Lastly, a longer observation period would be required to determine whether altered signaling observed in this study leads to the development of abnormal vascular structure and progressive vascular dysfunction.

In summary, this study demonstrates in a realistic, translational model of metabolic syndrome that insulin signaling through PI 3-kinase/Akt/eNOS versus ERK 1/2 MAP kinase pathways becomes imbalanced early in the course of diet-induced insulin resistance, accompanied by vascular dysfunction, but before any gross structural changes have occurred in the arterial wall. Increased expression of p85 α regulatory subunit of PI 3-kinase may play a role in the disruption of PI 3-kinase pathway signaling. Identification of endogenous early abnormalities of arterial signaling and function in insulin resistance may be important in designing therapeutic interventions to arrest or potentially reverse the progression of vascular dysfunction that currently affects a large segment of the population.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

In vivo and histologic vascular studies. **A** Systolic blood pressure (SBP) was measured in all pigs at baseline and again after 6 months of assigned diet. SBP was similar between groups at baseline and did not change significantly over 6 months in the standard diet (STD) group. However, at 6 months, SBP in the high fat high simple sugar diet (HFHS) group was significantly greater than baseline and significantly greater than STD group at 6 months. Diet x time interaction was also significant ($p < 0.05$). **B** Femoral artery flow-mediated vasodilatation (FMD) was measured in 8 pigs in each diet group at baseline and again after 6 months of assigned diet. Data are mean $\pm$ SE. FMD was similar in both groups at baseline and did not change over time in the STD diet group. However, at 6 months, FMD in the HFHS group was significantly decreased from baseline, and significantly lower than STD at 6 months. Diet x time interaction was also significant ($p < 0.05$). * $p < 0.05$. **C** Intima-media thickness as measured in the aorta was not different between STD ($n = 7$) and HFHS ($n = 7$) diet groups at 6 months. Horizontal lines and bars indicate mean and SE.

Figure 2.

PI 3-kinase and p85α expression in aortic lysates. **A** Insulin-stimulated IRS-1-associated PI 3-kinase activity in the aorta was measured by thin layer chromatography in STD (n=12) and HFHS (n=16) diet groups. Top: representative thin layer chromatography blot. Bottom: Group data, mean ± SE. In the absence of insulin stimulation, PI 3-kinase activity was similar in both diet groups. After exposure to an insulin bolus *in vivo*, the STD diet group exhibited the expected stimulation of PI 3-kinase activity. In contrast, this response was blunted in the HFHS diet group. The interaction of diet and insulin on PI 3-kinase activity was significant (p<0.001). **B** Expression of the p85α (regulatory) subunit of PI 3-kinase in aortic wall. Data are mean ± SE with n=15 (HFHS diet group) and n=14 (STD diet group). Total p85α expression was higher in the HFHS diet group compared with STD group.

*p<0.05, **p<0.001

Figure 3.

Expression of Ser473 phospho-Akt, total Akt and Akt isoforms in aortic wall of STD (n=13) and HFHS (n=16) groups. **A** Ser473 phospho- and total non-isoform-specific Akt. In the absence of insulin stimulation, Ser473 phospho-Akt was greater in HFHS than STD. With *in vivo* insulin exposure, Ser473 phospho-Akt increased as expected in STD but did not increase further in HFHS. Total Akt content did not differ between diet groups, with or without *in vivo* exposure to insulin. Control was set at 100% for STD group in absence of insulin stimulation. Diet x insulin interaction was significant ($p<0.001$). Data are mean $\pm$ SE. ** $p<0.001$. **B** Ser473 phospho-Akt1 and total Akt1, **C** Ser473 phospho-Akt2 and total Akt2, **D** Ser473 phospho-Akt3 and total Akt3. For each isoform, representative immunoblots are shown above group data for STD (n=13) and HFHS (n=16). There was a

trend suggesting greater phosphorylation of Ser473 Akt1 in STD than in HFHS. Insulin had no significant effect on Akt1 phosphorylation in either group. There was a modest increase in Akt2 Ser473 phosphorylation in response to insulin, which approached statistical significance in STD ($p=0.07$). Ser473 phospho-Akt3 increased in response to insulin in STD ($p<0.05$) but not HFHS. There was a significant interaction of diet and insulin stimulation on phospho-Akt3 ($p<0.05$). The total content of each Akt isoform did not differ significantly between diet groups or in the absence or presence of insulin.

Figure 4.

Phosphorylated Ser 1177 and total eNOS protein in aortic lysates from pigs fed STD (n=13) or HFHS (n=16) diet in the absence or presence of insulin treatment *in vivo*. Data are mean $\pm$ SE. Representative immunoblots are shown above group data. There was significant interaction of diet and insulin treatment on Ser1177 phospho-eNOS ($p=0.01$). The data suggest an effect of insulin to stimulate Ser1177 phospho-eNOS in STD ($^{\circ}p=0.07$), but not in HFHS. Ser1177 phospho-eNOS was significantly different between diet groups in the presence of insulin treatment. Total eNOS expression did not differ between groups in the absence or presence of insulin. Insulin-stimulated Ser1177 phospho-eNOS bands in HFHS are in non-contiguous lanes of same blot, demarcated by a space within a double line. All other lanes are contiguous. * $p<0.05$.

Table 1

Characteristics of diet groups at 0 months and after 6 months of assigned diet

	STD diet n=16 (8 M, 8 F) ⁺		HFHS diet n=16 (8 M, 8 F) ⁺	
	0 mo	6 mo	0 mo	6 mo
Weight (kg)	16.2 ± 0.6	36.0 ± 1.0 [*]	17.1 ± 0.6	78.5 ± 2.8 ^{*†}
Fasting glucose (mmol/l)	2.5 ± 0.1	2.3 ± 0.1	2.6 ± 0.5	2.8 ± 0.1 [†]
Fasting insulin (μU mL ⁻¹)	2.5 ± 0.4	2.4 ± 0.2	2.2 ± 0.4	6.0 ± 1.2 ^{*†}
IVGTT area under curve for glucose (mmol/l x min)	376 ± 13	386 ± 23	388 ± 16	493 ± 34 ^{*†}
IVGTT area under curve for insulin (mU/l x min)	727 ± 68	978 ± 151	594 ± 79	1794 ± 223 ^{*†}
Total cholesterol (mmol/l)	1.9 ± 0.1	1.8 ± 0.1	1.8 ± 0.1	2.5 ± 0.1 ^{*†}
Triglycerides (mmol/l)	0.38 ± 0.05	0.27 ± 0.02 [*]	0.34 ± 0.03	0.49 ± 0.07 ^{*†}

Data shown as mean ±SE

STD: standard diet group; HFHS: high fat high sugar diet group

⁺M: male; F: female^{*}Significant difference between time points within diet group (p<0.05)[†]Significant difference between diet groups at 6 months (p<0.05)