Published in final edited form as:

Surgery. 2012 September ; 152(3): 414–422. doi:10.1016/j.surg.2012.06.014.

Vodka And Wine Consumption In A Swine Model Of Metabolic Syndrome Alters Insulin Signaling Pathways In The Liver And Skeletal Muscle

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Abstract

Background—The purpose of this study was to examine the effects of alcohol in the context of metabolic syndrome on insulin signaling pathways in the liver and skeletal muscle.

Methods—Twenty six Yorkshire swine were fed a hypercaloric, high-fat diet for 4 weeks then split into three groups: hypercholesterolemic diet alone (HCC n=9), hypercholesterolemic diet with vodka (HCVOD n=9) and hypercholesterolemic diet with wine (HCW n=8) for 7 weeks. Animals underwent intravenous dextrose challenge prior to euthanasia and tissue collection.

Results—HCC, HCVOD and HCW groups had similar blood fasting glucose level, liver function test, and BMI. Thirty and 60 minutes after dextrose infusion, HCVOD and HCW groups had significantly elevated blood glucose compared to HCC. HCW group had significantly elevated insulin compared to HCC. Immunoblotting in skeletal muscle demonstrated that alcohol up-regulates p-IRS1, IRS2, AKT, AMPK α , PPAR α , FoxO1, and GLUT4. In the liver, HCW had up-regulation of AKT, AMPK α , and GLUT4 compared to HCC. Skeletal muscle immunohistochemistry demonstrated increased sarcolemmal expression of GLUT4 in both alcohol groups compared to HCC.

Conclusions—Moderate alcohol consumption in a swine model of metabolic syndrome worsens glucose metabolism by altering activation of the insulin signaling pathway in the liver and skeletal muscle.

Introduction

Metabolic syndrome is a group of metabolic risk factors that increases the risk of developing cardiovascular disease and type 2 diabetes [1]. Studies have demonstrated that metabolic syndrome more than doubles the risk of an acute myocardial infarction [2–4]. It is estimated that one in five US adults have metabolic syndrome, and the prevalence of metabolic syndrome is on the rise [5]. Insulin resistance, defined as reduced insulin sensitivity or metabolic response to insulin, is a hallmark of metabolic syndrome and type 2 diabetes [6].

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One modifiable risk factor for developing diabetes and cardiovascular disease is alcohol consumption [7].

Numerous epidemiological studies have examined the effect of alcohol and cardiovascular disease, and have described a J-shaped relationship between alcohol consumption and overall morbidity and mortality. This is known as the “French Paradox”, that is, despite the high intake of saturated fat, there is a reduction in risk of cardiovascular disease by at least 40% in low to moderate drinkers (defined as 20–30g alcohol/day) compared to abstainers. This effect is reversed with chronic heavy alcohol consumption (defined as >60g alcohol/day) [8–9]. The cardioprotective affect of alcohol has been linked to increased HDL, inhibition of platelet aggregation, and improved insulin sensitivity. Chronic and excessive alcohol leads to hypertension, hyperlipidemia, and insulin resistance. Alcohol seems to have a dose dependent effect on insulin signaling and risk of cardiovascular disease.

The liver and skeletal muscle are highly sensitive to the effects of both alcohol and insulin, and are critically important in maintaining glucose homeostasis [10–11]. In a previous study conducted in our lab, we examined the effects of resveratrol, which is a polyphenol plant extract that is considered the “heart-healthy” component in red wine on insulin signaling. The study showed that pigs with metabolic syndrome and chronic coronary ischemia supplemented with resveratrol had improved glucose control by up regulating the insulin signaling pathway[12]. These findings corroborate rat and mouse studies, which have also demonstrated improved insulin sensitivity in animals supplemented with resveratrol[13–15].

Though it is clear that resveratrol alone improves insulin sensitivity, it is unclear if alcohol in combination with resveratrol has any added benefit in improving insulin sensitivity. At present there are no human or large animal studies that evaluate the effect of alcohol on insulin signaling in the context of metabolic syndrome. We developed a clinically relevant swine model of metabolic syndrome and chronic coronary ischemia to investigate the effects of alcohol with resveratrol (wine) and without resveratrol (vodka) on insulin signaling in the liver and skeletal muscle.

Methods

ANIMAL MODEL

Twenty-six Yorkshire miniswine (Parsons Research, Amherst, MA) were fed 500g/day of a high-cholesterol diet consisting of 4% cholesterol, 17.2% coconut oil, 2.3% corn oil, 1.5% sodium cholate, and 75% regular chow (Sinclair Research, Columbia, MO). After 4 weeks of a high-cholesterol diet, all animals underwent ameroid constrictor placement to the left circumflex artery to simulate conditions of chronic cardiac ischemia as described previously [16]. Postoperatively, the animals were split into 3 different groups according to diet supplementation for an additional 7 weeks. The control group was continued on a hypercholesterolemic diet alone (HCC) (n=9). The hypercholesterolemic vodka (HCVOD) and hypercholesterolemic wine (HCW) groups were supplemented with 112 mL of vodka (Rubinoff Vodka, Somerville, MA) (40% EtOH/V, n=9) and 375 mL of red wine daily (2009 Pinot Noir, Black Mountain Vineyard, Napa and Sonoma, CA) (12.5% EtOH/V, n=8) respectively. Resveratrol content of the red wine was 0.3–0.5 µg/mL and was quantified by laser chromatography-mass spectroscopy. After 7 weeks of diet supplementation, all animals were anesthetized and the heart was exposed. After obtaining functional cardiac and hemodynamic measurements, the animals were euthanized by exsanguination and heart, liver and skeletal muscle samples were collected. Tissue samples were rapidly frozen in liquid nitrogen.

Animals were weighed at the time of diet initiation, after 4 weeks of high-cholesterol diet, and after 7 weeks of high cholesterol diet with supplementation. All animals were observed to ensure complete consumption of food and supplement, had unlimited access to water, and were housed in a warm non-stressful environment for the duration of the experiment.

The Institutional Animal Care and Use Committee of the Rhode Island Hospital approved all experiments. Animals were cared for in compliance with the “Principles of Laboratory Animal Care” formulated by the National Society for Medical Research and the “Guide for the Care and Use of Laboratory Animals” (NIH publication no. 5377-3 1996).

SEROLOGIC STUDIES

Blood samples were drawn from the jugular vein prior to euthanasia and tissue harvest. Blood glucose measurements were taken at baseline (fasting) and 30 and 60 minutes after an intravenous 0.5mg/kg dextrose infusion. Blood samples were also drawn and analyzed for insulin levels and liver function including aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and albumin. Liver enzymes were measured as a marker of liver function 4 weeks after initiation of a high fat diet, and 7 weeks after diet supplementation with alcohol. The chemistry laboratory at the Rhode Island Hospital, Providence, RI, analyzed serum samples.

PROTEIN EXPRESSION

Frozen tissue samples were homogenized and 40 micrograms of the RIPA (Radio-Immunoprecipitation Assay, Boston BioProducts, Ashland, MA) supplemented with protease and phosphatase inhibitors. Soluble fraction of whole-tissue lysates were fractionated by SDS-PAGE 3–8% Tris-Acetate gel (NuPage Novex Mini Gel, Invitrogen, Carlsbad, CA) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). Membranes were incubated overnight at 4°C with primary antibodies at dilutions recommended by the manufacturer against phosphorylated insulin receptor substrate 1 (p-IRS1) (Ser612), IRS2, phosphorylated AKT (Thr 308), AMP- activated protein kinase (AMPK α), FoxO1 (all from Cell Signaling, Danvers, MA), peroxisome proliferating activated receptor α (PPAR α) (Cayman Chemical, Ann Arbor, MI), and glucose transporter type 4 (GLUT4) (Epitomics, Burlingame, California). Membranes were incubated with the appropriate horseradish peroxidase-linked secondary antibody for one hour at room temperature (Jackson ImmunoResearch, West Grove, PA). Immune complexes were visualized with enhanced chemiluminescence and images were captured with a digital camera system (G-Box, Syngene, Cambridge, England). Band densitometry was quantified as arbitrary light units using Image-J software (National Institutes of Health, Bethesda, MD). All membranes were probed with α -tubulin to correct for loading error.

IMMUNOHISTOCHEMICAL ANALYSIS

Frozen liver and skeletal muscle was sectioned (12- μ m-thickness) and fixed in 10% formalin for 10 minutes. Sections were blocked with 1% bovine serum albumin in phosphate buffered saline for 1 hour at room temperature and incubated with anti-Glut 4 antibody (Epitomics) overnight at 4°C. Sections were then incubated with DyLight 488-conjugated anti-rabbit antibody (Jackson ImmunoResearch) for 45 minutes, then mounted with Vectashield with 4',6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). Images were captured at $\times 20$ magnification with a Nikon E800 Eclipse microscope (Nikon, Tokyo, Japan) at the same exposure. Brightness was enhanced identically in all images to optimally display immunofluorescence (Adobe Photoshop, San Jose, CA).

HEMATOXYLIN-EOSIN, PERIODIC ACID-SCHIFF, OIL RED O STAINING

Hematoxylin-Eosin, Periodic Acid-Schiff and Oil Red O staining was performed on frozen tissue sections by the Pathology and Histology core facility at Rhode Island Hospital. Images were captured at X20 magnification with a Nikon E800 Eclipse microscope (Nikon) from each representative tissue section in a random fashion. Histology analysis was conducted in a non-blinded fashion. H&E stained tissue was analyzed for necrosis, inflammatory cell infiltrate, cirrhosis or hepatocellular morphologic differences between groups.

DATA ANALYSIS

All results are reported as mean $\pm$ standard error of the mean. A one-way ANOVA was used to compare the means between groups followed by a post-hoc Bonferroni test using GraphPad Prism 5.0 Software (GraphPad Software Inc., San Diego, CA).

Results

ANIMAL MODEL

All animals were included in the analysis survived the entire experiment. One animal in the HCW group died intraoperatively of ventricular arrhythmia and another 4 in the HCC group died of intraoperative ventricular arrhythmia (1 animal) or of post-operative bleeding (3 animals). The animals that did not survive were excluded from analysis and replaced with new animals. Prior to euthanasia and tissue collection, the HCC, HCVOD and HCW groups had similar body mass index (BMI) (35.02kg/m², 32.44kg/m² and 33.17kg/m² respectively p=0.10).

SEROLOGIC STUDIES

At baseline, the HCC, HCVOD, HCW groups all had similar fasting serum glucose levels within normal limits (67, 63, and 66 mg/dl respectively). Thirty minutes after dextrose infusion, the HCVOD and HCW groups had significantly higher serum glucose compared to the control (207, 210, and 177 mg/dL respectively; p<0.05). At 60 minutes, the HCVOD and HCW serum glucose remained significantly higher (125mg/dL p<0.05 and 150mg/dL respectively; p<0.001) compared to control (105 mg/dL) (Figure 2). Fasting serum insulin levels were significantly higher in the HCW group compared to the HCC (Figure 1).

All three groups had similar levels of markers of liver function including ALT, AST, ALP and albumin at 4 weeks after initiation of high fat diet, and 7 weeks after diet supplementation with alcohol (p=0.24, p=0.89, p=0.89, and p=0.08 respectively) (Figure 2).

SKELETAL MUSCLE PROTEIN EXPRESSION

Western blot analysis in skeletal muscle demonstrated a marked increase in p-IRS1 in HCVOD compared to HCC, and HCVOD compared to HCW. There was also an increase in IRS2 in HCVOD compared to HCC and HCVOD compared to HCW. AKT was significantly elevated in the HCVOD and HCW groups compared to HCC. AMPK α was also elevated in the HCVOD and HCW compared to HCC. PPAR α was elevated in HCW compared to HCC and in HCVOD compared to HCW. FoxO1 was elevated in both HCW and HCVOD compared to HCC. GLUT4 was elevated in HCVOD compared to HCC (Figure 3).

LIVER PROTEIN EXPRESSION

Expression of AKT was elevated in HCW compared to HCC and HCVOD. AMPK α was up-regulated in HCW compared to HCVOD and HCC. GLUT4 expression was also elevated in HCW compared to HCVOD and HCC (Figure 4).

IMMUNOHISTOCHEMISTRY OF SKELETAL MUSCLE

The HCC skeletal muscle had the least amount of staining for membrane bound GLUT4. HCW and HCVOD had intense staining for membrane bound (sarcolemmal) GLUT4. There was more intense GLUT4 staining in the HCVOD skeletal muscle compared to HCW (Figure 5).

IMMUNOHISTOCHEMISTRY OF LIVER

In the liver, GLUT4 was primarily located in the cytoplasm, and demonstrated similar staining intensity among HCC, HCVOD and HCW groups (Figure 6).

SKELETAL MUSCLE HISTOLOGIC ANALYSIS

There were no morphologic differences among the three groups when stained with hematoxylin-Eosin. Periodic acid-Schiff staining was heterogeneous in the HCC group but homogeneous and similar in both HCVOD and HCW groups (Figure 5).

LIVER TISSUE HISTOLOGIC ANALYSIS

Hematoxylin-Eosin staining of the liver did not demonstrate any morphologic differences among the three groups. Periodic acid-Schiff staining was similar in intensity in all three groups. The HCVOD group demonstrated very little lipid deposition on oil red-O staining, whereas the HCC and HCW groups had increased amounts of hepatic lipid deposition (Figure 6).

Discussion

Yorkshire swine fed a high calorie/high-fat diet supplemented with moderate doses of alcohol demonstrated insulin resistance and up-regulation of the insulin signaling cascade compared to animals that were not supplemented with alcohol. Although all three animal groups had similar normal baseline blood glucose, at thirty and sixty minutes after dextrose infusion, the alcohol groups had significantly elevated blood glucose levels compared to HCC, suggesting that the alcohol groups had impaired glucose clearance. The alcohol groups also had elevated fasting insulin levels compared to HCC, although only the HCW group was significantly elevated. Though the HCVOD and HCW animals were not diabetic, they shared features of insulin resistance including decreased glucose clearance and elevated serum insulin levels, which are hallmarks of type 2 diabetes.

The HCVOD and HCW swine did not have any aversion to ingesting alcohol and all animals completed their alcohol soaked chow every day without issue. Also, there were no observed behavior differences between the animals consuming alcohol and the control animals. Serum alcohol levels were measured at the time of sacrifice and reported in a previously published manuscript [19]. Blood alcohol levels were similarly elevated in both the HCW and HCVOD swine one hour after eating and there was no significant difference in the blood alcohol content between the HCW and HCVOD groups. Serum resveratrol levels in the HCW group was not measured as the resveratrol content in the wine itself was very low (0.3–0.5 μ g resveratrol/mL wine) [12].

Animals that were fed hypercaloric/hypercholesterolemic diet supplemented with alcohol had similar BMIs compared to HCC despite the fact that they had higher caloric intake with the alcohol supplementation. This is likely due to the fact that they were only supplemented with alcohol over the course of 7 weeks. Perhaps if they were supplemented with alcohol over a longer period of time, they may have gained more weight based on the increased caloric intake compared to HCC.

The insulin resistance and obesity demonstrated in the current study is consistent with results we previously reported that Yorkshire swine fed a hypercaloric diet develop features of metabolic syndrome including obesity, insulin resistance, hypertension and dyslipidemia [17–18].

All animals had similar liver function tests suggesting that the dose of alcohol and or the duration of alcohol supplementation did not affect liver function. This was further confirmed with the H&E stained liver sections and there was no evidence of hepatocellular injury in the HCVD or HCW groups.

This study demonstrates that in a swine model of metabolic syndrome, moderate doses of alcohol in combination with a hypercaloric/hypercholesterolemic diet impairs glucose tolerance and alters the insulin signaling cascade. When insulin binds the insulin receptor, it results in tyrosine phosphorylation of IRS, which allows IRS to associate with phosphoinositide 3-kinase (PI3K). PI3K activates AKT, a serine kinase, which activates glycogen synthase thereby increasing glycogen synthesis. Activated AKT triggers the translocation of GLUT4 vesicles to the plasma membrane, where GLUT4 takes up glucose into the cell and reduces blood glucose levels[17]. In a well-fed state, AMPK α and mTOR are activated, which both in turn further activate the insulin signaling pathway. IRS also activates PPAR α , which is involved in lipid homeostasis and increases insulin sensitivity.

In skeletal muscle, alcohol increases serine phosphorylation of IRS1, which inhibits IRS1 from associating with PI3K. Alcohol up-regulates IRS2, AKT, AMPK α , and GLUT4, which propagate insulin signaling. Alcohol increases FoxO1, which is a member of the forkhead box transcription factor family and is important in regulating glycolysis and lipogenesis in the liver and skeletal muscle in a fasted state [18]. AKT phosphorylates and inactivates FoxO1 excluding it from the nucleus. In a fasted state, insulin levels are low, and FoxO1 is active promoting gluconeogenesis, protein turnover and amino acid catabolism. In a fed state, insulin levels are high, and AKT is activated and inactivates FoxO1 by excluding it from the nucleus. FoxO1 inhibition through the insulin signaling pathway suppresses gene expression involved in hepatic glucose production, amino acid catabolism and promotes glucose utilization and lipid synthesis. These results suggest that alcohol up-regulates FoxO1 expression, and this may in part explain the persistent glucose intolerance in a well-fed state.

Alcohol also up-regulates PPAR α , which is a member of the PPAR nuclear receptors that is expressed in the liver, heart, and skeletal muscle and is important regulator of fatty acid catabolism. When PPAR α is activated, it induces fatty acid uptake in the liver thereby reducing fatty acid inhibition of insulin signaling and improving insulin sensitivity [19]. Despite the alcohol induced upregulation of PPAR α , insulin resistance was still observed in these animals.

Steatohepatitis is a known complication of chronic ethanol consumption due to prolonged lipid accumulation, impaired lipid metabolism, lipotoxicity, inflammation and oxidative stress [21]. The earliest stage of steatosis is manifest by benign triglyceride accumulation in the liver. Interestingly, although there was increased hepatic lipid deposition in the HCC and

HCW groups, there was a paucity of hepatic lipid deposition in the HCVOD group. This may be due to preserved or improved lipid metabolism in the HCVOD group.

Periodic acid-Schiff staining in the liver demonstrated similar amounts of glycogen stores among all three groups. In the skeletal muscle, the HCC group appeared to have a more heterogeneous distribution of glycogen where the dark fibers are type 2/fast fibers and the light fibers are type 1/slow fibers. The alcohol groups demonstrated homogeneous glycogen stores and decreased dark type 2 fibers compared to HCC. This may be due to the fact that alcohol is preferentially toxic to type 2 fibers due to differential susceptibility to oxidative stress, and impaired protein synthesis and post-translational modification [22].

An interesting observation is that wine and vodka both up-regulated the same targets in the insulin signaling pathway and that both groups demonstrated glucose intolerance. We would have expected that the resveratrol-containing red wine would improve insulin sensitivity compared to vodka given the known anti-diabetic effects of resveratrol [13]. However, unlike the our previous study where animals were supplemented with resveratrol 100mg/kg/day, the resveratrol content in the wine given to the HCW group was much less (0.3–0.5 µg resveratrol/mL wine) [12].

In the liver, alcohol has a modest effect in amplifying insulin signaling by up-regulating AKT, AMPK α , and GLUT4. There was no apparent difference in GLUT4 expression in the liver and notably, it was mostly located in the cytosol. By contrast, in the skeletal muscle, GLUT4 was predominantly sarcolemmal and up-regulated in the HCVOD and HCW groups. Although many of the analytical differences of individual insulin signaling proteins demonstrated in this study are relatively small, the cumulative effect resulted in up-regulation of the insulin signaling cascade, as evidenced by the marked increase of GLUT4 up-regulation and translocation to the plasma membrane in the skeletal muscle. Despite the up-regulation of proteins involved in the insulin signaling pathway and increased membrane bound GLUT4 in the skeletal muscle in the alcohol supplemented groups, there was still persistently elevated serum glucose in the HCVOD and HCW groups and elevated serum insulin levels in the HCW group compared to HCC. The GLUT4 up-regulation to the plasma membrane did not result in the expected improved glucose uptake and insulin sensitivity in the HCVOD and HCW animals. This suggests that despite elevated GLUT4 levels at the plasma membrane, it may not be functional. In addition to translocation to the plasma membrane, GLUT4 must also be activated in order to allow glucose to enter the cell [20]. Perhaps high doses of alcohol inhibit the activation of GLUT4 at the plasma membrane.

Studies have shown that individuals who consume low to moderate quantities of alcohol (20g/d or 2 drinks/day) have lower risk of developing diabetes compared to abstainers, and consuming chronically high quantities of alcohol (>60g/d or >5drinks/day) increases the risk of developing diabetes [23]. The dose dependent effect of alcohol is complex and not fully understood. Animal studies have shown that low levels of alcohol improve insulin sensitivity by inhibiting gluconeogenesis by up-regulating AKT, reduces serum glucose by up-regulating GLUT4, reduces inflammation, up-regulates insulin sensitizers such as adiponectin, and increases insulin production by the pancreas [13, 22, 24]. High levels of alcohol consumption worsen insulin sensitivity by reducing ligand binding to the insulin receptor and decreasing activation of IRS and its down-stream targets including PI3K, AKT and GLUT4 [13, 22]. Perhaps the dose given in this study, 45g/d, represents the “tipping point” at which the anti-diabetic benefits of alcohol is reversed and tips the balance towards the development of insulin resistance and diabetes in the setting of metabolic syndrome. Further investigation at varying doses of alcohol in a swine model is warranted to elucidate the dose-response relationship between alcohol consumption and insulin signaling.

Acknowledgments

Funding

Funding for this research was provided by the National Heart, Lung, and Blood Institute (R01HL46716, R01HL69024, and R01HL85647, Dr. Sellke), NIH Training grant 5T32-HL094300-03, Drs Chu and Elmadhun), NIH Training grant 5T32-HL076134 (Dr. Lassaletta).

ABBREVIATIONS

HCC	High Cholesterol Control
HCVOD	High Cholesterol Vodka
HCW	High Cholesterol Wine
AST	Aspartate aminotransferase
ALT	Alanine aminotransferase
ALP	Alkaline phosphatase
p-IRS1	Phosphorylated insulin receptor substrate 1 (Ser612)
IRS2	Insulin receptor substrate 2
p-AKT	Phosphorylated AKT (Thr 308)
AMPKα	AMP- activated protein kinase
PPARα	Peroxisome proliferating activated receptor α
GLUT4	Glucose transporter type 4

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Figure 1. Fasting blood insulin and dextrose challenge

HCW group had a significantly elevated fasting insulin level compared to HCC. All three animal groups had similar baseline blood glucose (BG) levels. At 30 and 60 minutes after the dextrose infusion, HCVOD and HCW groups had significantly elevated BG compared to HCC. *p<0.05 HCC vs HCVOD; ** p<0.05 HCC vs HCVOD; †p<0.05 HCC vs HCW; ‡p<0.001 HCC vs HCW.

Figure 2. Liver Function Test

Markers of liver function were measured after 4 weeks of a high cholesterol diet and after 7 weeks of diet supplementation with alcohol. All three groups had similar liver function tests.

Figure 3. Expression of proteins involved in insulin signaling in skeletal muscle

Levels represent fold change $\pm$ standard error of the mean compared to HCC. Western blot analysis demonstrated that alcohol consumption increased expression of p-IRS1, IRS2, AKT, AMPK α , PPAR α , Fox01, and GLUT4.

Figure 4. Expression of proteins involved in hepatic insulin signaling

Levels represent fold change $\pm$ standard error of the mean compared to HCC. Western blot analysis demonstrated that alcohol consumption moderately increased expression of AKT, AMPK α , and GLUT4.

Figure 5. Skeletal Muscle Tissue Analysis

Row A: Periodic acid-Schiff staining in HCC, HCVOD and HCW respectively from left to right. Dark fibers seen in HCC are type 2/fast fibers and the light fibers are type 1/slow fibers. There is heterogeneous glycogen staining in the HCC group, and heterogeneous and similar amounts of glycogen staining in the HCVOD and HCW groups. Row B: Immunofluorescence staining for GLUT4. Red Staining represents GLUT 4 and blue staining represents nuclei. The HCC group has the least amount of GLUT 4 staining whereas HCVOD and HCW groups has more intense staining for membrane bound GLUT4. Row C: H&E staining. There are no differences in morphology among groups.

Figure 6. Liver Tissue Analysis

Row A: Oil red O staining in HCC, HCVD and HCW respectively from left to right. HCC and HCW have elevated levels of hepatic lipid deposition compared to HCVD. Row B: Periodic acid-Schiff staining. There are similar amounts of glycogen deposition in the liver among all three groups. Row C: Immunofluorescence staining for GLUT4. Red staining represents GLUT4 and blue staining represents nuclei. GLUT4 is primarily located in the cytosol and there are similar levels of GLUT4 staining among all three groups. Row D: H&E staining. There are no differences in morphology among groups.