Diet-induced obesity prevents interstitial dispersion of insulin in skeletal muscle

Running Title: Fat feeding reduces insulin access to skeletal muscle

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**Objective:** Obesity causes insulin resistance, which has been interpreted as reduced downstream insulin signaling. But, changes in access of insulin to sensitive tissues like skeletal muscle may also play a role. Insulin injected directly into skeletal muscle diffuses rapidly through the interstitial space to cause glucose uptake. When insulin resistance is induced by exogenous lipid infusion, this interstitial diffusion process is curtailed. Thus the possibility exists that hyperlipidemia, such as that seen during obesity, may inhibit insulin action to muscle cells, and exacerbate the insulin resistance. Here we asked whether interstitial insulin diffusion is reduced in physiological obesity induced by a high fat diet.

**Research design and methods:** Dogs were fed a regular diet (lean) or one supplemented with bacon grease for 9-12 weeks (high fat diet, HFD). Basal insulin (0.2mU/min/kg) euglycemic clamps were performed on fat-fed animals (n=6). During clamps done under anesthesia, 5 sequential doses of insulin were injected into the vastus medialis of one hindlimb (INJ); the contra-lateral limb (NINJ) served as a control.

**Results:** INJ lymph insulin showed an increase above NINJ in lean animals, but no change in HFD. Muscle glucose uptake observed in lean animals did not occur in HFD.

**Conclusions:** Insulin resistance induced by HFD caused a failure of intra-muscularly injected insulin to diffuse through the interstitial space and failure to cause glucose uptake, compared to normal animals. High fat feeding prevents the appearance of injected insulin in the interstitial space, thus reducing binding to skeletal muscle cells and glucose uptake.
Insulin resistance is associated with a number of diseases, including cardiovascular disease, hypertension(1), cancer(2) and obesity(1;3), as well as type 2 diabetes. Much research into insulin resistance has focused on insulin signaling, suggesting that target cell (e.g., skeletal muscle) response to insulin is the primary defect. Most recently, mitochondrial dysfunction(4;5) and intracellular fat accumulation(6) have been proposed as primary causes of insulin resistance. Despite these efforts, there is no definitive conclusion as to the primary cause of cellular insulin resistance.

There are a number of loci where insulin action can be altered, including the direct effect of inflammatory cytokines to attenuate distribution of blood flow and glucose uptake(7), and direct effects of plasma free fatty acids (FFAs) to impede insulin signaling in the cell(4) and/or reduce capillary recruitment(8). To act, insulin must cross the capillary endothelium, traverse the interstitial fluid compartment, thence access and bind to insulin receptors. A reduction in insulin’s ability to diffuse through the interstitial space in lipid-induced insulin resistance(9) suggests that access to sensitive tissues (such as skeletal muscle) may be important in addition to cellular insulin resistance. In insulin resistant situations, it is therefore important to examine whether insulin can access cells prior to stimulating cell signaling.

A high-fat diet, which induces obesity and leads to deposition of fat in tissues(10;11) does not always increase basal FFAs in humans or canines(12;13); but large elevations of FFAs can be observed at night in canines(13) and humans(14). Given uncertainty regarding the effect of fat diets on FFA levels in the circulation, it is not yet known whether effects of lipid infusion on insulin action can be extrapolated to explain how a high fat diet per se would affect insulin action and the ability of insulin to diffuse through skeletal muscle.

Previous studies in our laboratory have shown that insulin injected directly into healthy muscle is disseminated throughout the interstitial space, where the hormone accesses myocytes and stimulates glucose uptake(15). In a model of insulin resistance where plasma FFA was raised by lipid infusion, injected insulin was surprisingly not detected in the interstitial space (lymph) of skeletal muscle, supporting absence of elevated interstitial insulin. Under this condition there was little stimulation of glucose disposal(9). This latter result suggests that high plasma FFAs may prevent insulin’s access to skeletal muscle, resulting in insulin resistance. It cannot be concluded whether this is a direct effect of elevated plasma FFAs, or from the acute insulin resistance induced by lipid infusion. Therefore, it was important to examine whether lipid elevated physiologically by a high fat diet would similarly limit insulin’s access to muscle cells. For example, in dogs fed a control diet, insulin stimulates the extravascular distribution of insulin, without effects on vascular distribution volume(16), suggesting that insulin can increase its own access to insulin sensitive tissues. This effect was lost after induction of insulin resistance by a high fat diet(17) possibly due to an alteration in the diffusion of insulin through the interstitial space. In the present report, we examined whether insulin resistance induced by a high fat diet would simulate lipid infusion studies, in which injected insulin was not detected in lymph. If so, this would implicate insulin access to muscle cells as an important factor in dietary fat-induced insulin resistance.

**RESEARCH DESIGN AND METHODS**

**Animals:** One-year old male mongrel dogs were housed in individual cages in the
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University of Southern California Medical School Vivarium, and were kept under controlled conditions (12h light:12h dark). Animals had free access to water, and were fed once per day from 9:00-10:00am with 825grams dry chow (60% Prolab Canine 2000, 40% Laboratory High Density Canine Diet 5L18; PMI Nutrition International Inc. Brentwood, MO), and 1 can of Purina Pro-Plan Puppy Classic (Nestle Purina PetCare Company; St Louis, MO). For fat feeding, animals were subjected to the basal diet supplemented with 6 grams/kg bacon grease, presented to the dogs for the remainder of the study (15.2±0.8 weeks). We have previously published data from injecting insulin into normal-weight dogs (lean) fed ad lib dry chow (15); these previous results are reproduced with permission herein for comparison, and were not different when performed before or after the HFD experiments. Dogs were used for experiments only if they were judged to be in good health as determined by visual observation, body weight, hematocrit, and body temperature. Protocols were approved by the USC Institutional Animal Care and Use Committee, and were in accordance with the Principles of Laboratory Animal Care.

Study Design: Weekly fasted (basal) plasma samples for measurement of FFA were taken into tubes with 50µl EDTA and paraoxon to inhibit lipase activity, centrifuged, and the plasma frozen before analysis using a colorimetric kit (NEFA-HR(2), Wako Diagnostics, VA). Prior to beginning the high fat diet, each animal (n=6) received magnetic resonance imaging (MRI) of the abdominal area as outlined below, and a hyperinsulinemic euglycemic clamp (EGC) for determination of insulin sensitivity (SI) and glucose turnover. Once these baseline parameters were established, the high fat diet was administered, and the MRIs and EGCs were repeated (weeks 12-13). Finally, an acute injection procedure was performed prior to sacrifice to determine the local dynamics of injected insulin as detailed below. Lean animals received the acute injection procedure, but did not receive MRIs or EGCs.

Magnetic Resonance Imaging (MRI): On the morning of each MRI scan, a subcutaneous injection of Acepromazine (0.04mg/kg, Biocentual; St Joseph, MO) and Atropine Sulfate (0.1mg/kg, 1/120 grain, Western Medical; Arcadia, CA) was administered for preanaesthesia; dogs then received intravenous anesthesia of Ketamine HCL (10mg/kg, Phoenix Pharmaceutical; St Joseph MO) and Diazepam (0.2mg/kg, Abbott Laboratories, North Chicago). Body composition was then assessed in thirty 1-cm axial abdominal images using 1.5 Tesla Horizon Magnet (version 5.7 software). Each MRI slice was analyzed using Scion Image Software (Windows 2000 version Beta 4.0.2; Scion Corporation; Frederick, MD), which quantifies fat tissue (pixel values 121-254) and non-fat tissue (pixel values 20-120). The total pixels of fat and non-fat tissue were used to calculate the area of fat located within the peritoneal cavity in an 11cm region of the thorax identified by the central landmark slice, where the left renal artery branches from the abdominal aorta. Subcutaneous fat volume was calculated as total fat in the assessment region minus the visceral fat volume.

Hyperinsulinemic Euglycemic Clamp (EGC) Studies: A primed infusion of [3-H]-D-glucose tracer was started at t=-120minutes (25µCi bolus + 0.25 µCi/minute; DuPont-NEN; Boston, MA) into the saphenous vein in conscious animals. An infusion of insulin (0.75mU/min/kg; Lilly; Indianapolis, IN) and somatostatin (1.0µg/min/kg; Bachem California; Torrance, CA) was initiated at time 0, and radiolabelled dextrose (2.0µci/g, [3-H]-D-glucose, 50% dextrose) was variably infused to maintain euglycemia and specific activity. Blood samples were drawn from the cephalic vein...
every ten minutes from t=-30 to 180 min. Euglycemia was defined as the average basal glucose, and steady state as the final 30 minutes of the clamp. Insulin sensitivity ($S_I$) was calculated using the equation $S_I = \Delta G_{\text{inf}}/\Delta I_g$, where $\Delta G_{\text{inf}}=$change in glucose infusion at steady state from basal, $\Delta I_g=$change in plasma insulin at steady state from basal, and $G=$steady state plasma glucose concentration.

**Acute Insulin Injection Studies:**

Surgery. These experiments were terminal studies performed under anesthesia. Studies were done on animals, and on animals given 15.2±0.8weeks HFD. Animals were fasted 15hours before the experiment which began at 0600hrs. They were preanesthetized with acepromazine maleate (Prom-Ace, Aueco, Fort Dodge, IA; 0.22mg/kg) and atropine sulfate (Western Medical, Arcadia, CA; 0.11mL/kg). Anesthesia was induced with sodium pentobarbital (Western Medical, Arcadia, CA; 0.44mL/kg) and maintained with isofluorane (Western Medical, Arcadia, CA). Dogs were placed on heating pads to maintain body temperature. Indwelling catheters were inserted into the right jugular vein for a continuous saline drip (~1L for the first 60minutes of surgery and a slow drip thereafter) and into the left carotid artery for sampling and blood pressure monitoring (Space Labs, Issaquah, WA; model #90603A). Intracatheters were inserted into the left cephalic vein for variable glucose infusion and the right cephalic vein for insulin and somatostatin infusion. Indwelling catheters were also placed into both the right and left femoral arteries and veins for blood sampling. Two perivascular ultrasonic flowprobes (2mm diameter; Transonic, Ithaca, NY) were placed around both right and left femoral arteries proximal to the femoral catheter. Left and right hindlimb lymphatic vessels were cannulated by placing polyethylene catheters (PE10) into the afferent lymphatic vessels of the deep inguinal lymph node. Blood pressure, heart rate, $O_2$ saturation and $CO_2$ were monitored continuously. At the conclusion of these experiments, animals were euthanized with an overdose of sodium pentobarbital (Eutha-6, Western Medical; 65mg/kg).

**Blood glucose control during insulin injection.** Immediately after starting surgical procedures ($t=\text{180min}$), simultaneous systemic infusions of somatostatin (1ug/min/kg) and basal insulin replacement (0.2mU/min/kg) were begun and continued for the duration of the study (Fig. 1). Exogenous 20% glucose was infused at variable rates to maintain euglycemia at the basal level based on glucose measurements from the injected leg’s (INJ) femoral artery plasma glucose (measured prior to insulin infusion) throughout the entire experimental period ($t=\text{-240 to 300minutes}$). Samples were taken simultaneously from the right and left femoral arteries and veins. Left and right hindlimb lymph vessels were sampled by gently massaging the hindlimb distal to the site of catheterization from 2 minutes prior to 2 minutes after each blood sample.

**Unilateral intramuscular insulin injection.** As previously described(15), at times 0, 60, 120, 180 and 240 minutes, porcine insulin (0.3, 0.5, 0.7, 1, and 3U, represented as I1, I2, I3, I4, and I5 respectively) was injected directly into the vastus medialis of the quadriceps femoris of one leg. At each time point, two 30 gauge needles with a volume of 0.5mL per syringe injected the appropriate concentration of insulin at approximately 0.6mL/min. For individual experiments, either right or left hindlimb was chosen at random for injections (INJ) while the non-injected contra-lateral limb (NINJ) was used for comparison. Plasma and lymph samples were taken every 10minutes after each injection.

**Assays.** Plasma and lymph samples were collected in microtubes that were pre-coated with lithium-heparin (Becton
Dickinson, Franklin Lakes, NJ) and assayed for insulin and glucose. Arterial and venous tubes also contained 50uL EDTA (Sigma Chemicals). Blood samples were centrifuged immediately, and the supernatant was transferred and stored at -20°C until further assay. INJ femoral artery plasma samples were immediately assayed for glucose with an YSI 2700 auto analyzer (Yellow Springs Instrument Co., Yellow Springs, OH) before freezing at -20°C. Lymph samples were stored at -20°C after sampling. Samples were assayed for insulin by ELISA (Linco Research) adapted for dog plasma with a standard provided by Novo Nordisk, and glucose with an YSI 2700 auto analyzer.

Calculations. Glucose uptake across each limb was calculated based on Fick’s principle: $\text{LGU} = (\text{G}_A - \text{G}_V) \times \text{BF}$, where LGU = local glucose uptake; $\text{G}_A$ = Arterial glucose; $\text{G}_V$ = Venous glucose; BF = Femoral blood flow.

Statistical Analyses: All experimental data are expressed as the means ± SE. Statistical analyses were performed with unpaired Student’s t tests, one- or two-way ANOVAs with Tukey’s pairwise comparisons as appropriate. The lean and HFD groups were compared using unpaired t-tests and two-way ANOVAs.

RESULTS

14 weeks of fat feeding caused an increase in body weight, as shown in Figure 2A, and also resulted in an increase in both subcutaneous and abdominal fat, as measured by MRIs, shown in Figure 2B. On average, fat fed dogs increased visceral fat by 79.4% (from 462.5+81.4cm$^3$ to 803.2+154.3cm$^3$, P=0.02), and subcutaneous fat by 108.9% (from 902.2+183.1cm$^3$ to 1952.3+502.1cm$^3$, P=0.03).

Basal fasted plasma FFA decreased with fat feeding from 0.66+0.11mM to 0.40+0.07mM (P=0.02). During the course of the experiment, the plasma FFA levels dropped to levels virtually undetectable by the FFA assay used (data not presented), as inhalant isoflurane anaesthesia is known to suppress lipolysis (18).

Systemic insulin action: As expected, HFD induced significant peripheral insulin resistance determined by the conscious hyperinsulinemic euglycemic clamp (week 0: 8.72+0.27 to week 12: 3.15+0.65dL/pM/mg/kg, P=0.02, Figure 3A). Glucose disposal (Rd) decreased from 11.49+1.53mg/min/kg prior to fat feeding to 7.26+1.55mg/min/kg after 12 weeks of HFD (P=0.03, Figure 3B). Endogenous glucose production showed a trend to increase (week 0: 0.18+0.27 to week 12: 0.69+0.41mg/min/kg, P=0.36, Figure 3C).

HFD caused the dogs to become insulin resistant, and this was more evident in the periphery than in the liver.

Local insulin action – lean animals: As previously reported, insulin injections caused a small increase in both INJ and NINJ femoral artery insulin over the course of the experiment (INJ: 0min, 11.9+1.0; 300min, 35.9+1.9mU/L, P<0.001. NINJ: 0min, 12.4+1.2; 300min, 36.6+2.5mU/L, P<0.001, Figure 4A). Femoral vein insulin was significantly increased after the final injection in INJ compared to NINJ (300min: NINJ, 35.8+2.0; INJ, 64.0+10.5mU/L, P=0.02, Figure 4B), demonstrating a washout of insulin injected into the muscle, which then entered the systemic circulation to cause the small elevations in arterial insulin. INJ lymph insulin concentrations increased after the second injection when compared to NINJ, and remained significantly elevated until the end of the experiment (300min: NINJ, 13.2+2.3; INJ, 73.8+7.1mU/L, P<0.01, Figure 4C). The concentration of insulin in the interstitial fluid of NINJ remained approximately half of that of the arterial insulin supply. Insulin injection into muscle induced an increase in glucose infusion required to maintain euglycemia in lean animals, as well as a significant increase...
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**Local insulin action – Fat fed animals:** In HFD animals, in sharp contrast to lean animals, INJ lymph insulin concentrations showed no elevation in lymph insulin during injection into the muscle when compared to the contralateral non-injected limb (300min: NINJ, 20.9+1.4; INJ, 26.6+4.9mU/L, P=0.232. Figure 4C). The minor increase in femoral artery insulin over time was observed similar to lean animals and not different between legs (INJ: 0min, 15.7+1.1; 300min, 47.6+2.4mU/L, P<0.001. NINJ: 0min, 17.3+1.4; 300min, 46.8+3.2mU/L, P<0.001, Figure 4A). INJ femoral vein insulin was significantly increased compared to NINJ (300min: HFD NINJ, 49.6+5.55, HFD INJ, 133.9+22.9mU/L, P=0.03), and lean INJ (300min: HFD INJ, 133.9+22.9; lean INJ, 64.0+10.5mU/L, P=0.01) reflecting a significantly greater washout of insulin from the injected area in HFD compared to lean animals from 210 min to the end of the experiment (Figure 4B). The interstitial fluid insulin concentration in NINJ was approximately half the arterial insulin concentration; this was not significantly different compared to lean animals, and did not change throughout the experiment. The glucose infusion for fat fed animals was not significantly different from lean animals, however the femoral artery glucose was significantly higher than in the lean animals, due to a higher basal glucose measured prior to insulin and somatostatin infusion (Lean: 96.0+2.1mg/dl, HFD 11.6+4.8mg/dl, P=0.01, unpaired t-test). There was no significant glucose uptake due to insulin injection (300min: INJ 5.9+7.14, NINJ 4.68+6.2mg/min/leg, P=0.328), which was significantly lower than in lean animals (P=0.02, Figure 5). There was no significant difference in blood flow (Figure 6), mean arterial pressure (lean: 63.82+3.01, HFD: 68.83+3.56mmHg) or heart rate (lean: 110.09+4.79, HFD: 111.67+5.72bpm) between groups.

**DISCUSSION**

Physiological insulin resistance induced by feeding a high fat diet for 14 weeks caused a failure of insulin injected into skeletal muscle to be detected in lymph, and prevented glucose uptake in response to the injection compared to lean animals. This is reminiscent of previously published results that show experimentally-elevated plasma lipids likewise inhibit the ability of injected insulin to diffuse through muscle and initiate glucose uptake(9). Interestingly, the ability of the HFD to impair insulin dispersion in muscle occurs in the absence of a rise in plasma free fatty acids, either under basal fasting conditions, or during the clamp.

There was no significant difference between the injected and non-injected arterial insulin in the fat-fed animals, as the arterial supply to both legs should be the same and the insulin injection occurs distal to the arterial sampling point. Also, there is no significant difference between the femoral artery and vein insulin concentrations of the non-injected leg, so the increase observed in the femoral vein insulin concentration of the injected leg was due to insulin overflow from the local area of the injection. Amongst the high fat fed animals, there was variation in the ability of lymph to reflect the more concentrated insulin injections. As the ratio of NINJ lymph to arterial insulin was maintained in the face of increasing systemic insulin, the ability of lymph to detect whole body insulin changes was not altered.

Lymph measures of interstitial insulin have previously been used in this laboratory(9;15-17;19:20) and others(21-24), and agree with other measures of interstitial insulin such as microdialysis(25). Further, gold injected into the interstitium has been
shown to concentrate in lymph vessels(26). Due to the low flow rate and apparent lack of size exclusion of lymph vessels, rapid equilibration of lymph with the intracellular fluid can occur similar to that utilized in microdialysis, and as such is an appropriate tool for measuring interstitial insulin concentrations, reflecting the makeup of the interstitial fluid to which myocytes are exposed. One limitation in our study is that the lymph vessel insulin concentration is a mixture across the entire leg, whereas insulin is injected into a small area of one muscle; thus the interstitial concentration of insulin in the injected area may be higher than in lymph due to dilution of lymph from uninjected muscle. However, previous results have shown a significant increase in lymph insulin in lean dogs(15), thus HFD has impaired the appearance of insulin in lymph.

We show that the ability of insulin to access myocytes is impaired in physiological insulin resistance, and several proposed causes of insulin resistance are unable to describe the results we observe here. For example, mitochondrial dysfunction has been proposed as a critical component of insulin resistance(27), yet in our study, cells are not exposed to elevated insulin, and as such, mitochondrial dysfunction is unlikely to be responsible for the impaired dispersion of insulin. Studies in obese mice have shown that mitochondrial dysfunction is evident in adipose tissue, but not in skeletal muscle(28), and while stimulating FFA oxidation in skeletal muscle reduces insulin resistance(29), it does not necessarily follow that mitochondrial function is the primary cause of skeletal muscle insulin resistance. Similarly, it is unlikely that downstream signaling impairment in myocytes(30;31) or intracellular fat accumulation(6;32) impair glucose uptake in our study, as there was apparently no measurable elevation of interstitial insulin to cause insulin’s cellular effects. However, we can not discount that myocytes would exhibit insulin resistance if the cells were actually exposed to injected insulin in our model.

The mechanism(s) by which HFD prevents injected insulin from appearing in the interstitial space can not be clarified from the experiments presented here. As acute hyperlipidemia also resulted in reduced insulin appearance in the interstitial fluid(9), it is unlikely to be a structural change. One possibility is a lipid-induced alteration in permeability of the endothelial cell(33;34), which might allow a more rapid egress of insulin from the interstitial compartment into the bloodstream. However, this permeability theory does not identify the location of the injected insulin prior to appearance in the vein, which may occur as much as one hour later. It was noted that, while insulin was not detected in lymph, venous insulin had not dropped to pre-injection levels even an hour after injection. As less than 30% of the injected insulin is removed by the vein during the course of the experiment, the remainder must in theory be contained within the muscle, although is not detected in the interstitial space. The excess may be taken up into some cells. Insulin is internalized by endothelial cells en route to the vein(35), and, as endothelial cells exhibit insulin resistance(36), this internalization may be increased in obesity to overcome the impaired insulin signaling, thus removing insulin from the interstitial space. As there is no insulin in the interstitial space, only myocytes immediately adjacent to the area of injection may internalize insulin. Alternatively, in lean animals, injected insulin could increase the perfusion of muscle and augment the blood flow to the area, therefore increasing the dispersion of insulin through the muscle(37). Obese rats(38) and those with pharmacologically elevated lipid(8) have impaired insulin-mediated capillary recruitment, insulin injected into the muscle may be trapped in the small area of injection,
without the means to increase its dispersion area. It is also possible that the local high insulin concentration insulin after injection is in the interstitial space, although not in an area sampled by the lymph. In lean animals, injected insulin would cause an increase in the distribution volume, to allow more complete dissemination of insulin through the muscle, and therefore detection in the lymph. Adiposity would prevent this increase in distribution volume(39), and reduce the appearance of insulin in the lymph, as less of the muscle is exposed to insulin.

The role of the endothelial cell in the transport of insulin is unclear – evidence exists for both para- and trans-cellular transport of insulin. A recent study has shown that in cell culture, endothelial cells concentrate insulin(38); whether this occurs in vivo has not been determined, and there is no indication of whether this is unidirectional or can move from plasma to interstitium and back. As lean animals show significant insulin appearance in the vein, insulin can move in both directions. While endothelial cells exhibit insulin resistance(36), effects on insulin transport are not known, though insulin resistance can impair the appearance of insulin in the interstitium(20;23), and transport is known to be rate limiting to insulin action(19;20;22).

While we have shown that insulin resistance, either chronic (HFD) or acute(9), prevents dissemination of insulin through the muscle, the mechanism is not yet known. As the HFD does not increase, and in fact suppresses, basal plasma FFAs, and isoflurane anesthesia reduces plasma FFAs by 70%(17), plasma FFA are unlikely to be the primary cause of the impaired dissemination of injected insulin. However, as plasma lipids are elevated nocturnally by a high fat diet(13), it is possible that while plasma FFAs are not a direct cause of insulin resistance in our study, tissue FFA levels may be. Intramyocellular lipid content can be altered by diet(11) and acutely by lipid infusion(40); as many cells are not exposed to injected insulin it is unlikely that the intracellular lipid would affect our results, though there is the possibility of extracellular fat accumulation. It is unclear whether lipid must be in the interstitial space to inhibit dispersion of intramuscularly injected insulin, or whether another factor may be inhibiting the dispersion of insulin.

The HFD dogs were subjected to a significantly higher glucose level than the lean animals throughout the injection procedure, as their plasma glucose level after anesthesia and prior to insulin and somatostatin infusion was higher. The arterial glucose of animals infused with lipid is not significantly different to either lean or HFD animals, and values appear to be intermediate(9). Endothelial dysfunction may be caused by both acute hyperglycemia(41) and hyperlipidemia(42), which may prevent insulin induced capillary recruitment. Thus, impaired insulin dispersion observed with lipid infusion(9) and fat feeding may result from different plasma factors that ultimately affect the capillary endothelium. Further studies are necessary to determine whether elevated glucose affects the dispersion of injected insulin.

In conclusion, in a high fat diet, which induces chronic insulin resistance, insulin injected directly into the muscle is not detectable in the interstitial space (lymph), but appears to wash out in the venous blood flow. This is similar to previous results(9) which demonstrate acute lipid-induced insulin resistance prevented injected insulin from diffusing through the interstitium. The apparent cellular insulin resistance occurs because myocytes are not exposed to insulin; potential downstream effectors such as mitochondrial dysfunction, intramyocellular fat accumulation and signaling impairment are unlikely to be responsible for the inability of injected insulin to diffuse through the
interstitial space in this diet-induced insulin resistance. While the injection study is not a physiological situation, there are clear differences in how insulin is dealt with in the interstitium, and its access to muscle. Therefore, studies on the insulin sensitivity of individual cells should consider whether insulin was able to access the tissue prior to any perceived defect in insulin signaling or response.

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REFERENCES
1. Natali, A, Ferrannini, E: Hypertension, insulin resistance, and the metabolic syndrome. Endocrinol Metab Clin North Am. 33:417-429, 2004
2. Jee, SH, Kim, HJ, Lee, J: Obesity, insulin resistance and cancer risk. Yonsei Med J. 46:449-455, 2005
3. Reaven, GM: Insulin resistance: the link between obesity and cardiovascular disease. Endocrinol Metab Clin North Am. 37:581-viii, 2008
4. Morino, K, Petersen, KF, Shulman, GI: Molecular mechanisms of insulin resistance in humans and their potential links with mitochondrial dysfunction. Diabetes. 55 Suppl 2:S9-S15. S9-S15, 2006
5. Anderson, EJ, Lustig, ME, Boyle, KE, Woodlief, TL, Kane, DA, Lin, CT, Price, JW, III, Kang, L, Rabinovitch, PS, Szeto, HH, Houmard, JA, Cortright, RN, Wasserman, DH, Neufer, PD: Mitochondrial H2O2 emission and cellular redox state link excess fat intake to insulin resistance in both rodents and humans. J Clin Invest. 37048, 2009
6. Virkamaki, A, Korsheninnikova, E, Seppala-Lindroos, A, Vehkavaara, S, Goto, T, Halavaara, J, Hakkinen, AM, Yki-Jarvinen, H: Intramyocellular lipid is associated with resistance to in vivo insulin actions on glucose uptake, antilipolysis, and early insulin signaling pathways in human skeletal muscle. Diabetes. 50:2337-2343, 2001
7. Youd, JM, Rattigan, S, Clark, MG: Acute impairment of insulin-mediated capillary recruitment and glucose uptake in rat skeletal muscle in vivo by TNF-alpha. Diabetes. 49:1904-1909, 2000
8. Clerk, LH, Rattigan, S, Clark, MG: Lipid infusion impairs physiologic insulin-mediated capillary recruitment and muscle glucose uptake in vivo. Diabetes. 51:1138-1145, 2002
9. Chiu, JD, Kolka, CM, Richey, JM, Harrison, LN, Zuniga, E, Kirkman, EL, Bergman, RN: Experimental hyperlipidemia dramatically reduces access of insulin to canine skeletal muscle. Obesity (Silver Spring). 17:1486-1492, 2009
10. Krebs, M, Roden, M: Molecular mechanisms of lipid-induced insulin resistance in muscle, liver and vasculature. Diabetes Obes Metab. 7:621-632, 2005
11. St Onge, MP, Newcomer, BR, Buchthal, S, Aban, I, Allison, DB, Bosarge, A, Gower, B: Intramyocellular lipid content is lower with a low-fat diet than with high-fat diets, but that may not be relevant for health. Am J Clin Nutr. 86:1316-1322, 2007
12. Golay, A, Chen, YD, Reaven, GM: Effect of differences in glucose tolerance on insulin's ability to regulate carbohydrate and free fatty acid metabolism in obese individuals. J Clin Endocrinol Metab. 62:1081-1088, 1986
13. Kim, SP, Catalano, KJ, Hsu, IR, Chiu, JD, Richey, JM, Bergman, RN: Nocturnal free fatty acids are uniquely elevated in the longitudinal development of diet-induced insulin resistance and hyperinsulinemia. Am J Physiol Endocrinol Metab. 292:E1590-E1598, 2007
14. Tasali E, Broussard J, Day A, Delebecque F, van Cauter E: Nocturnal free fatty acid levels are elevated after sleep restriction in healthy young adults (Abstract). Diabetes 58: Suppl 1, 2009
15. Chiu, JD, Richey, JM, Harrison, LN, Zuniga, E, Kolka, CM, Kirkman, EL, Ellmerer, M, Bergman, RN: Direct administration of insulin into skeletal muscle reveals that the transport of insulin across the capillary endothelium limits the time course of insulin to activate glucose disposal. Diabetes 57:828-835, 2008
16. Ellmerer, M, Kim, SP, Hamilton-Wessler, M, Hucking, K, Kirkman, E, Bergman, RN: Physiological hyperinsulinemia in dogs augments access of macromolecules to insulin-sensitive tissues. *Diabetes.* 53:2741-2747, 2004

17. Ellmerer, M, Hamilton-Wessler, M, Kim, SP, Huecking, K, Kirkman, E, Chiu, J, Richey, J, Bergman, RN: Reduced access to insulin-sensitive tissues in dogs with obesity secondary to increased fat intake. *Diabetes.* 55:1769-1775, 2006

18. Horber, FF, Krayer, S, Miles, J, Cryer, P, Rehder, K, Haymond, MW: Isoflurane and whole body leucine, glucose, and fatty acid metabolism in dogs. *Anesthesiology.* 73:82-92, 1990

19. Ader, M, Richey, JM, Bergman, RN: Evidence for direct action of alloxan to induce insulin resistance at the cellular level. *Diabetologia.* 41:1327-1336, 1998

20. Yang, YJ, Hope, ID, Ader, M, Bergman, RN: Insulin transport across capillaries is rate limiting for insulin action in dogs. *J Clin Invest.* 84:1620-1628, 1989

21. Castillo, C, Bogardus, C, Bergman, R, Thuillez, P, Lillioja, S: Interstitial insulin concentrations determine glucose uptake rates but not insulin resistance in lean and obese men. *J Clin Invest.* 93:10-16, 1994

22. Castillo, CE, Lillioja, S: Peripheral lymphatic cannulation for physiological analysis of interstitial fluid compartment in humans. *Am J Physiol.* 261:H1324-H1328, 1991

23. Miles, PD, Levisetti, M, Reichard, D, Khoursheed, M, Moossa, AR, Olefsky, JM: Kinetics of insulin action in vivo. Identification of rate-limiting steps. *Diabetes* 44:947-953, 1995

24. Miles, PD, Li, S, Hart, M, Romeo, O, Cheng, J, Cohen, A, Raafat, K, Moossa, AR, Olefsky, JM: Mechanisms of insulin resistance in experimental hyperinsulinemic dogs. *J Clin Invest.* 101:202-211, 1998

25. Sjostrand, M, Holmang, A, Lonroth, P: Measurement of interstitial insulin in human muscle. *Am J Physiol.* 276:E151-E154, 1999

26. SHERMAN, AI, TER POGOSSIAN, M: Lymph-node concentration of radioactive colloidal gold following interstitial injection. *Cancer.* 6:1238-1240, 1953

27. Lowell, BB, Shulman, GI: Mitochondrial dysfunction and type 2 diabetes. *Science.* 307:384-387, 2005

28. Choo, HJ, Kim, JH, Kwon, OB, Lee, CS, Mun, JY, Han, SS, Yoon, YS, Yoon, G, Choi, KM, Ko, YG: Mitochondria are impaired in the adipocytes of type 2 diabetic mice. *Diabetologia.* 49:784-791, 2006

29. Tanaka, T, Yamamoto, J, Iwasaki, S, Asaba, H, Hamura, H, Ikeda, Y, Watanabe, M, Magoori, K, Ioka, RX, Tachibana, K, Watanabe, Y, Uchiyama, Y, Sumi, K, Iguchi, H, Ito, S, Doi, T, Hamakubo, T, Naito, M, Auwerx, J, Yanagisawa, M, Kodama, T, Sakai, J: Activation of peroxisome proliferator-activated receptor delta induces fatty acid beta-oxidation in skeletal muscle and attenuates metabolic syndrome. *Proc Natl Acad Sci USA.* 100:15924-15929, 2003

30. Kim, YI, Lee, FN, Choi, WS, Lee, S, Youn, JH: Insulin regulation of skeletal muscle PDK4 mRNA expression is impaired in acute insulin-resistant states. *Diabetes.* 55:2311-2317, 2006

31. Yu, C, Chen, Y, Cline, GW, Zhang, D, Zong, H, Wang, Y, Bergeron, R, Kim, JK, Cushman, SW, Cooney, GJ, Atcheson, B, White, MF, Kraegen, EW, Shulman, GI: Mechanism by which fatty acids inhibit insulin activation of insulin receptor substrate-1 (IRS-1)-associated phosphatidylinositol 3-kinase activity in muscle. *J Biol Chem.* 277:50230-50236, 2002
32. Bachmann, OP, Dahl, DB, Brechtel, K, Machann, J, Haap, M, Maier, T, Loviscach, M, Stumvoll, M, Claussen, CD, Schick, F, Haring, HU, Jacob, S: Effects of intravenous and dietary lipid challenge on intramyocellular lipid content and the relation with insulin sensitivity in humans. *Diabetes.* 50:2579-2584, 2001

33. Dell'Omo, G, Penno, G, Pucci, L, Mariani, M, Del Prato, S, Pedrinelli, R: Abnormal capillary permeability and endothelial dysfunction in hypertension with comorbid Metabolic Syndrome. *Atherosclerosis.* 172:383-389, 2004

34. St Pierre, P, Bouffard, L, Papirakis, ME, Maheux, P: Increased extravasation of macromolecules in skeletal muscles of the Zucker rat model. *Obesity.(Silver.Spring).* 14:787-793, 2006

35. Wang, H, Wang, AX, Liu, Z, Barrett, EJ: Insulin signaling stimulates insulin transport by bovine aortic endothelial cells. *Diabetes.* 57:540-547, 2008

36. Gogg, S, Smith, U, Jansson, PA: Increased MAPK activation and impaired insulin signaling in subcutaneous microvascular endothelial cells in type 2 diabetes: the role of endothelin-1. *Diabetes.* 58:2238-2245, 2009

37. Clark, MG, Wallis, MG, Barrett, EJ, Vincent, MA, Richards, SM, Clerk, LH, Rattigan, S: Blood flow and muscle metabolism: a focus on insulin action. *Am J Physiol Endocrinol Metab.* 284:E241-E258, 2003

38. Wallis, MG, Wheatley, CM, Rattigan, S, Barrett, EJ, Clark, AD, Clark, MG: Insulin-mediated hemodynamic changes are impaired in muscle of Zucker obese rats. *Diabetes.* 51:3492-3498, 2002

39. de Jongh, RT, Ijzerman, RG, Serne, EH, Voordouw, JJ, Yudkin, JS, de Waal, HA, Stehouwer, CD, van Weissenbruch, MM: Visceral and truncal subcutaneous adipose tissue are associated with impaired capillary recruitment in healthy individuals. *J.Clin.Endocrinol.Metab.* 91:5100-5106, 2006

40. Brechtel, K, Dahl, DB, Machann, J, Bachmann, OP, Wenzel, I, Maier, T, Claussen, CD, Haring, HU, Jacob, S, Schick, F: Fast elevation of the intramyocellular lipid content in the presence of circulating free fatty acids and hyperinsulinemia: a dynamic 1H-MRS study. *Magn Reson.Med.* 45:179-183, 2001

41. Nieuwdorp, M, van Haeften, TW, Gourveneur, MC, Mooij, HL, van Lieshout, MH, Levi, M, Meijers, JC, Holleman, F, Hoekstra, JB, Vink, H, Kastelein, JJ, Stroes, ES: Loss of endothelial glycocalyx during acute hyperglycemia coincides with endothelial dysfunction and coagulation activation in vivo. *Diabetes.* 55:480-486, 2006

42. de Jongh, RT, Serne, EH, Ijzerman, RG, de Vries, G, Stehouwer, CD: Free fatty acid levels modulate microvascular function: relevance for obesity-associated insulin resistance, hypertension, and microangiopathy. *Diabetes.* 53:2873-2882, 2004
Fat feeding reduces insulin access to skeletal muscle

FIGURE LEGENDS

**Figure 1.** Euglycemic basal insulin clamp protocol. Time in minutes is shown across the top. Somatostatin was infused with basal insulin replacement, glucose was infused at a variable rate to maintain euglycemia based on the INJ arterial glucose measurement. Samples were taken at 15 minute intervals from -60 to 0 minutes, and at 10 minute intervals from time 0-300 from the femoral artery, femoral vein and lymph, of INJ and NINJ legs. I1, I2, I3, I4 and I5 represent insulin injections of 0.3, 0.5, 0.7, 1.0 and 3.0 units respectively which occurred every 60 minutes from time 0.

**Figure 2.** (A) Dog weights for lean animals, and animals prior to (white) and after (black) fourteen weeks of a high fat diet (HFD). There is no significant difference between lean animals and week 0 measurements for animals subjected to a high fat diet. (B) MRIs were performed prior to (Week 0, white bars) and after a high fat diet (Week 14, black bars). Fat feeding induced an accumulation of both visceral and subcutaneous adipose tissue, *, P<0.05 using paired t-test.

**Figure 3.** Changes in (A) SI, (B) Rd, and (C) EGP as calculated from hyperinsulinemic EGCs prior to (Week 0, white bars) and after 14 weeks (black bars) of HFD. *, significantly different to corresponding Week 0, P<0.05 (paired t-test).

**Figure 4.** Insulin concentration in femoral artery (A), femoral vein (B) and lymph (C). Lean animals are included, shown in the left column, compared to new data from animals fed a high fat diet shown in right column. In both columns, black squares represent data from INJ leg, white squares represents data from NINJ leg, dotted vertical lines indicate the injection times. *, significantly different to all other groups (two way ANOVA). #, significantly different to corresponding contralateral leg (paired t-test).

**Figure 5.** Local glucose uptake in lean (A) and HFD (B) animals. INJ (black squares) LGU was significantly elevated above NINJ (white squares) in lean animals as marked, *, significantly different to NINJ (paired t-test). There was no significant difference between INJ and NINJ in HFD animals.

**Figure 6.** Blood flow effects in lean (circles) and HFD (squares) animals. INJ (black symbols) blood flow was not significantly different to NINJ (white symbols) at any time point, and did not change throughout the experiment (paired and unpaired t-test as appropriate).
Fat feeding reduces insulin access to skeletal muscle

Figure 1.

EUGLYCEMIC CLAMP

Time (minutes):
-180 -60 0 60 120 180 240 300
BASAL

Injections:
I1 I2 I3 I4 I5

Somatostatin + Basal Insulin
(1.0 ug/min/kg) (0.2 mU/min/kg)

Glucose (20%) Variable

Figure 2.

A

Body weight (kg)
Control
HFD

P=0.06

B

Fat tissue (cm^3)
VISCERAL
SUBQ

*
Fat feeding reduces insulin access to skeletal muscle

Figure 3.
Fat feeding reduces insulin access to skeletal muscle

Figure 4.
Figure 5.

Figure 6.