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Modest hyperglycemia prevents interstitial dispersion of insulin in skeletal muscle

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Abstract

Insulin injected directly into skeletal muscle diffuses rapidly through the interstitial space to cause glucose uptake, but this is blocked in insulin resistance. As glucotoxicity is associated with endothelial dysfunction, the observed hyperglycemia in diet-induced obese dogs may inhibit insulin access to muscle cells, and exacerbate insulin resistance. Here we asked whether interstitial insulin diffusion is reduced in modest hyperglycemia, similar to that induced by a high fat diet.

METHODS—During normoglycemic (100mg/dl) and moderately hyperglycemic (120mg/dl) clamps in anesthetized canines, sequential doses of insulin were injected into the vastus medialis of one hindlimb; the contra-lateral limb served as a control. Plasma samples were collected and analyzed for insulin content. Lymph vessels of the hind leg were also catheterized, and lymph samples were analyzed as an indicator of interstitial insulin concentration.

RESULTS—Insulin injection increased lymph insulin in normoglycemic animals, but not in hyperglycemic animals. Muscle glucose uptake was elevated in response to hyperglycemia, however the insulin-mediated glucose uptake in normoglycemic controls was not observed in hyperglycemia. Modest hyperglycemia prevented intra-muscularly injected insulin from diffusing through the interstitial space reduced insulin-mediated glucose uptake.

CONCLUSION—Hyperglycemia prevents the appearance of injected insulin in the interstitial space, thus reducing insulin action on skeletal muscle cells.

Keywords

glucose; interstitium; endothelium; skeletal muscle; insulin

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CMK designed experiments, CMK, AVBC, ELK performed experiments, CMK analyzed results, CMK and RNB wrote and edited manuscript.

CMK is guarantor for this work.

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1. INTRODUCTION

Obesity, insulin resistance and hypertension are all components of the metabolic syndrome, and the links between each of the diseases involved are continually being investigated. Elevated fasting plasma glucose ($>5.6\text{mmol/L}$) or impaired glucose tolerance are diagnostic criteria for the metabolic syndrome put forward by several expert groups, including the World Health Organization, the International Diabetes Federation, the National Cholesterol Education Program and others, and fasting hyperglycemia is noted as a step on the progression to diabetes, often occurring after impaired glucose tolerance¹. Raised fasting glucose levels are associated with impaired insulin secretion, while impaired glucose tolerance is thought to reflect insulin resistance².

Insulin action *in vivo* is frequently studied, as is insulin action at the cellular level *in vitro*. However the ability of insulin to get to the cell surface *in vivo* is essential for the cellular response to insulin, and there is a gradient in insulin concentration from the plasma to the interstitial space³⁻⁶. Insulin transport to the interstitial space is rate limiting for whole body insulin action^{5;7} and is impaired in insulin resistance⁸. Insulin injected into healthy muscle is rapidly detected within the interstitial space using lymph cannulation to sample interstitial fluid, and a similarly rapid effect on muscle glucose uptake can also be detected⁹. Insulin resistance induced by either acute lipid infusion or feeding of a high fat diet prevented insulin from reaching the interstitial fluid and prevented glucose uptake^{10;11}. While both treatments induce insulin resistance by excess lipid, in contrast to lipid infusion the fat feeding intervention does not induce elevated plasma lipid during the study, therefore the factor preventing the access of insulin to the interstitial space is not known. It was noted that the fat-fed dogs had a modestly higher plasma glucose level (120mg/dl compared to approximately 95mg/dl in controls) during the experiment¹¹; this was evident when the dogs were under anesthesia. Since this fasting glucose level is above that used in the diagnostic criteria for the metabolic syndrome, it is possible that the slight hyperglycemia could be responsible for limiting the access of insulin to the cell.

Glucotoxicity can have effects on many different cell types, and is known to cause endothelial dysfunction^{12;13}. As insulin's ability to access tissue relies on healthy responses to insulin in the blood vessel wall, such as vasodilation, capillary recruitment and transendothelial transport, hyperglycemia and the resulting endothelial dysfunction may therefore impair the dispersion of insulin through muscle. A recent study has shown that obese women with postprandial hyperglycemia have impaired insulin delivery to both adipose tissue and skeletal muscle¹⁴, an effect that may be due to the obesity or the postprandial hyperglycemia. Here we investigate the effect of modestly elevated glucose on the access of insulin to muscle. Surprisingly, we find that mild hyperglycemia is sufficient to impair the distribution of injected insulin through the interstitial space.

2. MATERIALS AND METHODS

2.1 Animals

Experiments were performed on anesthetized male mongrel dogs. Animals were housed in the University of Southern California Medical School Vivarium under controlled kennel

conditions (12 h light:12 h dark) and fed standard chow (49% carbohydrate, 25% protein, 9% fat; Alfred Mills, Chicago, IL) once per day. 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 University of Southern California Institutional Animal Care and Use Committee.

2.2 Groups

In an effort to conserve animals, some of the animals from our previous publications have been incorporated into the control group ⁹, however we have expanded this control group to ensure that previous results still apply; as such the data is not identical to previous publications. The two groups for comparison within this manuscript were those maintained at normoglycemic levels (control), and those maintained at hyperglycemic levels. The level of hyperglycemia was chosen to match that of previously published high fat fed animals, approximately 120mg/dl.

2.3 Protocol

Animals were fasted 15 hours before the morning of the experiment at 0600 hrs. Dogs were preanesthetized with acepromazine maleate (Prom-Ace, Aueco, Fort Dodge, IA; 0.22mg/kg) and atropine sulfate (Western Medical, Arcadia, CA; 0.11 mL/kg). Anesthesia was induced with sodium pentobarbital (Western Medical, Arcadia, CA; 0.44mL/kg) and maintained with isoflurane (Western Medical, Arcadia, CA). Indwelling catheters were implanted in the right jugular vein for a continuous saline drip (~ 1 L for the first 60 minutes of surgery and a slow drip thereafter) and in the left carotid artery for sampling and blood pressure monitoring (Space Labs, Issaquah, WA; model #90603A). Intracatheters were inserted into the cephalic veins for glucose, insulin, somatostatin and tracer infusion. Indwelling catheters were also placed into both the right and left femoral arteries and veins for sampling. Two perivascular ultrasonic flowprobes (2mm diameter; Transonic, Ithaca, NY) were placed around both right and left femoral arteries proximal to the femoral catheter for measuring rates of blood flow. Left and right hindlimb lymphatic vessels were cannulated by placing polyethylene catheters (PE10) into the afferent lymphatic vessels of the deep inguinal lymph node. Lymph was collected by gently massaging the leg directly above the popliteal area, which has been shown to instantaneously increase lymph drainage without affecting lymph or plasma oncotic pressures. Blood pressure, heart rate, O₂ saturation and CO₂ were monitored continuously. At the conclusion of these experiments, animals were euthanized with an overdose of sodium pentobarbital (Eutha-6, Western Medical; 65mg/kg).

2.4 Clamps

Immediately after starting surgical procedures, a basal insulin euglycemic clamp was started (t=-180min). Glycemia was maintained by variably infusing exogenous 20% glucose to maintain euglycemia (n=8) or hyperglycemia (120mg/dl)(n=7), glucose infusion rate was chosen to maintain euglycemia in the femoral artery plasma of the injected leg. Simultaneously, somatostatin was infused (1µg/min/kg; Bachem), and basal insulin replaced systemically (1.2pmol/min/kg; Novo Nordisk, Bagsvaerd, Denmark) and continuously for the remainder of the study (Fig. 1). 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 point.

2.5 Intramuscular insulin

At times 0, 60, 120, 180 and 240 minutes, porcine insulin (0.3, 0.5, 0.7, 1, and 3 U, represented as I1, I2, I3, I4, and I5 respectively) was injected into the vastus medialis of the quadriceps femoris with two 30 gauge needles with a volume of 0.5mL per syringe (Fig. 1). Each injection was given at approximately 0.6 mL/min. For individual experiments, right or left hindlimb was chosen at random for injections while the contra-lateral limb served as a control. Plasma and lymph samples were taken every 10 minutes after each injection.

2.6 Assays

Plasma and lymph samples were assayed for insulin and glucose. Arterial, venous and lymph samples were collected in microtubes that were pre-coated with lithium-heparin (Becton Dickinson, Franklin Lakes, NJ). Arterial and venous sample 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. Femoral artery plasma samples taken from the injected hindlimb were immediately assayed for glucose with an YSI 2700 autoanalyzer (Yellow Springs Instrument Co., Yellow Springs, OH) before freezing at -20°C . Lymph samples were immediately stored at -20°C after sampling. Porcine insulin was measured in plasma and lymph with an ELISA adapted for dog plasma, CV average of 2.7%.

2.7 Calculations

Glucose uptake across the limb was calculated based on Fick's principle: $\text{LGU} = (G_A - G_V) \cdot \text{BF}$, where LGU= local glucose uptake; G_A = Arterial glucose; G_V = Venous glucose; BF = Femoral blood flow. Values were averaged for the hour after each injection.

2.8 Statistical analyses

All experimental data in graphs and text are expressed as the means $\pm$ Standard Deviation. Statistical analyses were performed with unpaired Student's *t* tests or two-way ANOVAs with Tukey's pairwise comparisons as appropriate.

3. RESULTS

Upon entering the study, the body weight of the dogs in the control and hyperglycemia groups was not significantly different (control: $26.9 \pm 2.7\text{kg}$, $n=8$ vs hyperglycemia: $29.1 \pm 4.8\text{kg}$, $n=7$). As can be seen in Figure 2, the hyperglycemic group had a significantly higher plasma glucose concentration, as determined by the study design. Previous studies demonstrated that a high fat diet caused a significantly higher basal glucose level (lean 97.5 ± 7.8 , High Fat Diet $112.7 \pm 13.1\text{mg/dl}$)¹¹. These animals had been subjected to a 12 week high fat diet, and as such had significantly higher body weight ($34.0 \pm 7.41\text{kg}$, $n=6$, $P=0.024$ vs controls). The complete glucose profile in the fat-feeding study is shown in Figure 2A, and there is a significant difference between the lean and fat-fed groups. Figure 2B demonstrates the maintenance of significantly higher arterial glucose levels throughout

the current experiments in the hyperglycemic group, compared to a new control group. Interestingly, there was not a significant difference in the glucose infusion rates required to induce hyperglycemia compared to the control group (Figure 2C).

Glucose concentrations in the arterial plasma and lymph are shown in Figure 3. By design, the hyperglycemia group had a significantly higher glucose concentration in both artery and lymph than control experiments (figure 3). Lymph glucose concentrations are approximately 10% higher than arterial levels in the non-injected leg throughout most of the study, which is consistent with previous studies, and maintained with hyperglycemia in the early stages of the experiment. Interestingly, once insulin injections began, the interstitial glucose concentration in the injected leg reduced to match arterial glucose levels in both control and hyperglycemic conditions, possibly reflecting insulin-mediated glucose uptake. This effect is not observed in the contralateral leg until much later.

Plasma and lymph insulin concentrations are shown in Figure 4. There is no significant difference between the arterial insulin concentration entering the injected and the non-injected leg (figure 4A), and no difference between the control and hyperglycemic animals. After each injection, a substantial increase in vein insulin concentration of the injected leg is observed, significantly increased above the non-injected leg (Figure 4B). There is no significant difference between the control (left) and hyperglycemic (right) panels. In control animals (Figure 4C, left panel) lymph insulin concentration increases after each injection indicated by the vertical dotted line. However, with the same injection protocol into animals subjected to modest acute hyperglycemia, insulin injection does not increase lymph insulin. This suggests that the injected insulin is not accessing the cell.

At basal, blood flow rates were 143.8 ± 44.2 ml/min and 196.4 ± 64.3 ml/min in control injected and non-injected legs respectively, and at the end of the experiment these were 171.7 ± 52.5 and 198.8 ± 47.9 ml/min. In hyperglycemia, flow rates were 178.0 ± 78.6 and 161.0 ± 60.4 in injected and non-injected legs at basal, and 186.9 ± 41.6 and 185.3 ± 58.7 ml/min at the end of the experiment. Blood flow was therefore not significantly affected by either insulin injection or hyperglycemia.

Figure 5 shows the leg glucose uptake for each leg, averaged for the hour after injection. The control group has a significant increase in glucose uptake in the injected leg after insulin injection when compared to the basal time point, as well as to the non-injected leg at the same time point ($P < 0.05$, two way ANOVA). However there was no significant effect of insulin injection on leg glucose uptake under hyperglycemic conditions, suggesting that the significant increase in glucose uptake due to insulin injection in control animals is lost with hyperglycemia.

4. DISCUSSION

Here we show that even modest hyperglycemia can impair the dispersion of injected insulin through the interstitial space. While intramuscularly injected insulin enters the vein at the same rate in control and hyperglycemia, access of insulin to the interstitium (indicated by our lymph measurements) was impaired by modestly elevated plasma glucose suggesting that the injected insulin is unable to access the cell surface and is thus unable to initiate

insulin-mediated glucose uptake. This is remarkably similar to our previously published results in a fat-fed dog model¹¹, suggesting that the hyperglycemia observed in the obese dog may be responsible for the impaired access of insulin. Further, a recent study has shown that obese women with postprandial hyperglycemia exhibit an impaired delivery of insulin to both adipose tissue and skeletal muscle¹⁴. This is consistent with our results shown here, and we advance these findings as modest acute hyperglycemia is sufficient to reduce insulin access to skeletal muscle even when obesity is not present.

Glucotoxicity is already a major focus in diabetes research. Physiological hyperglycemia typically results from impaired insulin secretion^{2;15}, and modest acute hyperglycemia can inhibit first-phase insulin secretion¹⁶. However our results are independent of insulin release. Glucotoxicity can affect fat and muscle cells directly¹⁷ manifesting as a global downregulation of gene expression, and it has been hypothesized that cellular insulin resistance is due to hyperglycemia in adipocytes¹⁸. A study of cultured human skeletal muscle cells indicates that hyperglycemia alone can decrease glucose transport¹⁹. Thus, acute hyperglycemia may have prevented cellular insulin-mediated glucose uptake in our study. However we show the injected insulin does not appear to access the interstitial space, so while hyperglycemia may indeed cause cellular defects in insulin action, there is also reduced access to the cells.

It is interesting that a short term, mild increase in plasma glucose levels can have a significant effect on insulin sensitivity and access. The glycemia induced in this study was chosen to match that of the fat-fed animals during a similar experiment¹¹ -- a level lower than postprandial glucose levels in healthy subjects -- which would not be thought to induce insulin resistance. However this experiment induces a more prolonged hyperglycemia than is reasonably expected in postprandial situations as it is sustained for at least 6 hours. While this glucose concentration matches that often observed in diabetes or the metabolic syndrome, our experiment provides only acute glucose elevation, and therefore it is surprising that this relatively short exposure time is still able to significantly impair insulin access to the interstitial space. This finding may therefore have substantial relevance for patients with raised fasting glucose levels resulting from defective insulin secretion²: our results suggest a pathway by which elevated glucose can lead to insulin resistance, by preventing insulin access to tissues.

This study is designed to clarify insulin's local effects in muscle. Once in the interstitial space, insulin must be able to diffuse readily through the milieu surrounding the cells to bind cell surface receptors to initiate insulin signaling cascades leading to glucose uptake. Our previous studies have shown that this is rapid in healthy animals⁹, and that there is a clear impairment in the ability of injected insulin to access myocytes in insulin resistance^{10;11}: as hyperglycemia also reduces insulin access, it is possible that hyperglycemia was the cause of reduced insulin access in fat fed animals, and may have contributed to reduced insulin action. While hyperglycemia has been shown to have direct effects on cellular insulin sensitivity^{17;18}, we detect no insulin at the cell surface using the insulin concentration in lymph as a surrogate; further studies directly examining how hyperglycemia may affect the ability of insulin to move from the endothelium to the interstitium should be performed.

We have previously shown that insulin resistance induced both by fat-feeding and by lipid infusion can impair access of injected insulin to the interstitial space. Interestingly there is no obvious common factor that could be responsible for this similarity in response. Lipid infusion increases plasma lipid, yet there is no elevated plasma lipid during the experiment in fat fed animals. There is, however, a nocturnal increase in lipid with a high fat diet²⁰ which could cause fat incorporation in either the interstitial or intracellular space, and we intend to investigate whether intramyocellular or interstitial lipid can directly impair dispersion of insulin. However, by mimicking the elevated plasma glucose seen in fat-fed animals¹¹, we were able to demonstrate that hyperglycemia alone is sufficient to impair the dispersion of injected insulin through muscle. An interesting follow-up study could investigate whether blood glucose normalization in our previously published fat-fed model¹¹ would restore insulin access, which would suggest that impaired access is an acute response to hyperglycemia, not accumulation of fat. While other factors of the high fat diet may also impair endothelial function and hormone access, our finding that hyperglycemia alone appears to reduce interstitial access reinforces the impact of glucotoxicity and may suggest a direct benefit to lowering glucose levels in diabetes and obesity.

The common mechanism by which insulin appearance in the interstitium is impaired in hyperglycemia, fat-feeding¹¹ and lipid infusion¹⁰ remains to be established. Impairment of insulin access to the interstitial space is similar in these studies, in spite of the different methods of inducing insulin resistance. As hyperglycemia does not occur during lipid infusion¹⁰, we are left with the conclusion that there are at least two independent factors that impair access of injected insulin in skeletal muscle: hyperlipidemia and hyperglycemia. Therefore we can conclude that both lipotoxicity and glucotoxicity can affect insulin access to the interstitium in muscle. Endothelial dysfunction can be induced by both hyperglycemia²¹ and hyperlipidemia^{22;23}, and has been observed in type 2 diabetes^{24;25}, obesity²⁶ and prediabetes²⁷ as well as in hypertension with the metabolic syndrome²⁸.

Hyperglycemia can affect the endothelium in a variety of ways. Inflammation induced by hyperglycemia can cause vascular deterioration²⁹ due to the close relationship between blood glucose levels and microvascular function, and endothelial cell dysfunction can play a role in both the metabolic and vascular alterations in type 2 diabetes¹². Hyperglycemia increases permeability, simultaneously decreasing the number of perfused capillaries³⁰. Other studies have confirmed that glucotoxicity can be associated with both increased permeability of the endothelial barrier³¹ and endothelial dysfunction²¹. Increased permeability could indicate that injected insulin would be leaking out into the venous circulation, though this was not supported by our current observations.

Apart from permeability, appropriate endothelial function is an important component of insulin action. The hemodynamic effects of insulin increase delivery of insulin and other nutrients to the myocyte and other cell types and rely on appropriate vascular function^{32–34}. In healthy animals insulin is effectively dispersed through the muscle by insulin's own ability to increase its area of distribution³⁵, an effect impaired in cases of insulin resistance³⁶. We have previously discussed the potential requirement of healthy vascular responses to distribute even intramuscularly injected insulin¹¹, whereby insulin's effects to increase muscle perfusion^{32–34} are necessary to efficiently disseminate injected insulin

throughout the muscle. Thus, our findings of reduced interstitial insulin in skeletal muscle in cases of acute insulin resistance¹⁰, diet-induced insulin resistance¹¹, and now hyperglycemia may be due to impaired endothelial function: further investigation is required to determine whether endothelial function is in fact impaired in these states, and whether it may be the primary defect leading to reduced access.

Previously with insulin resistance there was a significant increase in vein plasma insulin^{10;11} suggesting an increased washout of the injected insulin. However, venous insulin after injection was not significantly different between hyperglycemia and controls, suggesting that hyperglycemia did not increase the washout of injected insulin. Since the amount of insulin in the lymph did not change there is a substantial amount of injected insulin that is so far unaccounted for. Analysis of previous results have shown that up to 80% of the injected insulin is unaccounted for at the conclusion of the experiment, thus the location of the injected insulin remains unknown. Some of the injected insulin may be trapped near the injection site, or concentrated in one particular cell type such as the endothelial cells *en route* to the plasma³⁷, but further investigation is required. We have considered using either fluorescent or radiolabel techniques to map the location of the injected insulin. Another possible explanation of our results is that the ability of lymph to reflect the interstitial environment is impaired in disease states, such that insulin is in the tissue, but not detected in lymph. While lymph has been proposed as a very effective method of sampling interstitial fluid¹⁵, we are investigating techniques to confirm lymph as an appropriate measure of interstitial fluid under a variety of conditions.

The ability to sample the muscle interstitium under such dynamic conditions is one of the strengths of this model. Other studies measure the interstitial fluid using microdialysis, which does not allow for complete recovery of insulin and provides a smaller sample than lymph. The lymph technique is minimally invasive compared to other interstitial sampling options, providing a representation of the unmanipulated *in vivo* environment. However, this study is limited by the lack of mechanistic data. Specific measures of microvascular dysfunction that may strengthen these results have not been established in the canine model, and biopsies that are taken at the conclusion of the experiment may be greatly influenced by the variable proximity to the insulin injection site. Further, assays for the canine model are often not well established making analysis of inflammation or protein expression changes difficult.

The fact that such a mild hyperglycemia can alter the local muscle environment may have substantial clinical implications. As hyperglycemia is an early effect during progression to diabetes, this could be a mechanism by which hyperglycemia eventually causes insulin resistance. Impaired insulin secretion leads to raised fasting glucose levels, and impaired glucose tolerance contributes to the development of insulin resistance². Our finding could link these effects, where even mild hyperglycemia can cause impaired insulin access, as well as potentially altered cellular insulin sensitivity, both of which are associated with insulin resistance. Studies have shown that treating postprandial hyperglycemia does not seem to slow progression to type 2 diabetes, though there was no effect on the fasting plasma glucose levels of these patients, which were maintained at approximately the same level as those in our study³⁸. Subjects who were able to return to fasting plasma glucose levels more

quickly after a glucose challenge had lower risk of type 2 diabetes 8 years later³⁹, which when combined with results shown here, may implicate the prolonged postprandial hyperglycemia as contributing to reduced insulin access and thus insulin resistance. Preventing mild hyperglycemia may be a treatment method to slow or prevent the progression of impaired pancreatic function to insulin resistance by maintaining appropriate insulin access to the muscle.

In conclusion, we demonstrate that modest increases in plasma glucose levels for only a short period of time are able to reduce the access of injected insulin to myocytes, as well as prevent insulin-injection mediated increases in local glucose uptake. The similarity to our previously published results implicate hyperglycemia as a possible cause for impaired insulin access to muscle and insulin resistance in the fat fed dog model¹¹. While the level of plasma glucose is similar to that used as a predictor of the metabolic syndrome, the exposure time is very short, in the order of a few hours. This suggests that even slight glucose intolerance may change insulin's fate in the interstitial space, highlighting the contribution of glucotoxicity to metabolic disease. We conclude that short term modest hyperglycemia is capable of impairing the access of insulin to skeletal muscle.

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Abbreviations

BF	blood flow
LGU	leg glucose uptake
PKC	protein kinase C

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GLUCOSE CLAMP

Figure 1. Basal insulin glucose 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 either euglycemia based on the injected leg arterial glucose measurement, or hyperglycemia (120mg/dl). 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 injected and non-injected 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. Glucose levels and glucose infusion rates

(A) Arterial glucose concentrations in lean animals (black symbols, n=9) were found to be significantly lower than animals fed a high fat diet (white symbols, n=6) in a previous study (data adapted from {Kolka, 2009 324/id}). (B) A new batch of lean controls (black symbols, CON, n=8) showed similar glucose levels to previously published lean animals, and the hyperglycemia group (white symbols, HG, n=7) were maintained at a significantly elevated arterial glucose level to match animals fed a high fat diet (Figure 2A). The control animals had significantly lower arterial glucose concentrations throughout the experiment. Both hyperglycemia and fat-fed groups were significantly different to control, $P < 0.01$ (Two way ANOVA). (C) Glucose infusion to attain the glucose levels shown in Figure 2B, no significant difference between lean (black) and hyperglycemic (white) animals. (*, $P < 0.05$, two way ANOVA)

Figure 3. Artery and lymph glucose

Glucose concentrations in artery (black symbols) and lymph (white symbols) of control (CON, squares, n=8) and hyperglycemia (HG, circles, n=7) experiments. Vertical dotted lines represent times of insulin injection (shown in Figure 1).

Figure 4. Insulin levels

Insulin concentrations in A) femoral artery, (B) femoral vein, and (C) lymph of control (left panel) and hyperglycemia (right panel) animals. Black symbols show the non-injected leg, and white symbols represent the injected leg, dotted vertical lines indicate the injection times. *, $P < 0.05$ (two way Anova) compared to non-injected leg in same group.

Figure 5. Leg glucose uptake

Glucose uptake change from basal for each hour after each injection in control (CON) and hyperglycemic (HG) animals. Control Injected leg was significantly different to all other groups ($P < 0.05$, Two-way ANOVA).