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## Variable Hepatic Insulin Clearance with Attendant Insulinemia is the Primary Determinant of Insulin Sensitivity in the Normal Dog

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### Abstract

**OBJECTIVE**—Insulin resistance is a powerful risk factor for Type 2 diabetes and a constellation of chronic diseases, and is most commonly associated with obesity. We examined if factors other than obesity are more substantial predictors of insulin sensitivity under baseline, non-stimulated conditions.

**DESIGN AND METHODS**—Metabolic assessment was performed in healthy dogs (n=90). Whole-body sensitivity from euglycemic clamps ( $SI_{CLAMP}$ ) was the primary outcome variable, and was measured independently by IVGTT (n=36). Adiposity was measured by MRI (n=90), and glucose-stimulated insulin response was measured from hyperglycemic clamp or IVGTT (n=86 and 36, respectively).

**RESULTS**— $SI_{CLAMP}$  was highly variable (5.9 to 75.9 dl/min per kg per  $\mu$ U/ml). Despite narrow range of body weight (mean, 28.7 $\pm$ 0.3 kg), adiposity varied  $\sim$ 8-fold and was inversely correlated with  $SI_{CLAMP}$  ( $p<0.025$ ).  $SI_{CLAMP}$  was negatively associated with fasting insulin, but most strongly associated with insulin clearance. Clearance was the dominant factor associated with sensitivity ( $r=0.53$ ,  $p<0.00001$ ), whether calculated from clamp or IVGTT.

**CONCLUSIONS**—These data suggest that insulin clearance contributes substantially to insulin sensitivity, and may be pivotal in understanding the pathogenesis of insulin resistance. We propose that hyperinsulinemia due to reduction in insulin clearance is responsible for insulin resistance secondary to changes in body weight.

### Keywords

Insulin Resistance; Insulin Clearance; Adiposity; Insulin Secretion; Obesity

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## Introduction

The widespread increase in prevalence of Type 2 diabetes in the U.S. and abroad has prompted efforts to identify persons at high risk for the disease in the hopes that early intervention may delay or prevent disease onset. One of the most common diabetes risk factors is insulin resistance. While resistance itself may be a heritable trait (1), obesity can further reduce insulin sensitivity. Yet even among lean subjects with normal glucose tolerance, insulin sensitivity can vary substantially (2), suggesting that other factors may be at play that determine sensitivity under baseline conditions.

In nondiabetic subjects, reduced insulin sensitivity is met with compensatory hyperinsulinemia which overcomes sluggish insulin action and maintains glucose tolerance and normal fasting glycemia. It has generally been thought that hyperinsulinemia was the result of upregulation of pancreatic  $\beta$ -cell sensitivity to glucose, and subsequent increase in secretion of insulin. However, we and others have shown (3,4) that the hyperinsulinemia characteristic of obesity and insulin resistance reflects the combined effects of increased insulin secretion and decreased metabolic clearance of the hormone, primarily by the liver. However, the reverse dynamic is also plausible, i.e. that reduction of insulin clearance is the primary event, which could generate hyperinsulinemia that would negatively feed back on insulin-sensitive tissues, thus generating insulin resistance. It remains unclear which physiologic process is the dominant driver that determines insulin sensitivity, particularly under baseline, overnight fasted conditions.

This study was undertaken to identify the major determinants of insulin sensitivity, using data from a sizable cohort of healthy, mongrel dogs. Our results indicate that insulin clearance is the primary determinant of insulin sensitivity under overnight-fasted conditions. We postulate that reduction in insulin clearance by the liver results in hyperinsulinemia which in turn, may lead to development of insulin resistance in the obese dog model. Thus we suggest that insulin resistance may be secondary, rather than primary in the development of insulin resistance secondary to increased storage of fat.

## Methods and Procedures

### Animal Model

Procedures were performed on 90 male mongrel dogs ( $28.7 \pm 0.3$  kg;  $\sim 1$  yr old). Data were pooled from previously published studies (5-12). Baseline data were obtained from overnight-fasted animals over a 2-3 week period, prior to enrollment in any pharmacologic or dietary treatment. All animals underwent MRIs to quantify total and regional adipose mass and a hyperinsulinemic euglycemic clamp (EGC) to assess insulin sensitivity. Subsets of these animals also underwent insulin-modified intravenous glucose tolerance tests (IVGTT;  $n=36$ ) or stepwise hyperglycemic clamps (STEP clamp;  $n=86$ ). An outline of the specific procedures performed on each dog is shown in Table 1.

At least one week prior to testing, dogs were surgically outfitted with a chronic catheter ( $n=49$ ) or sampling port ( $n=30$ ) in the jugular vein, advanced into the right atrium for sampling of mixed central venous blood. Blood sampling for remaining dogs ( $n=11$ ) was

performed using peripheral intracatheters inserted percutaneously into a limb vein. Dogs were individually housed in the vivarium under controlled environmental conditions, and fed a combination of dry chow and canned food to maintain stable body weight. Time interval for feeding varied amongst animals (from 1 hr feeding to ad lib regimens, but all testing was performed after an overnight fast, and we observed no effect of feeding pattern on any measured variable. All procedures were approved by the University of Southern California Institutional Animal Care and Use Committee.

## Experimental Procedures

The following procedures were performed after overnight fasting. During all procedures (except MRIs), dogs were fully conscious, resting comfortably in a Pavlov sling, and were given free access to water.

1. **MRI:** Dogs were pre-anesthetized and sedated. Thirty 1-cm axial abdominal images were obtained using a GE 1.5 Tesla Horizon magnet. Computer MRI images were analyzed by a skilled laboratory technician who was blinded to other measures of body composition of index animals. Analysis was performed using ScionImage software, which quantified fat tissue (pixel value: 0 to 120) and non-fat tissue (pixel value: 121 to 256) in each slice. Total trunk fat volume was estimated as the integrated fat across all 30 slices. Visceral fat was defined as fat within the peritoneal cavity at  $\pm 5$  image “slices” from the level where the left renal artery branches from the abdominal aorta (i.e. from 11-cm abdominal range). Adiposity was expressed in units of  $\text{cm}^3$ .
2. **Euglycemic Hyperinsulinemic Clamps:** On the morning of the clamp, two intracatheters were inserted percutaneously in each saphenous vein for infusions of glucose and HPLC-purified  $3\text{-}^3\text{H}$ -glucose (“tracer”). At  $t = -180$  min, a primed infusion of tracer ( $25 \mu\text{Ci} + 0.25 \mu\text{Ci}/\text{min}$ ; Perkin-Elmer NEN) was initiated, and tracer continued for a 90 min equilibration period. After basal blood sampling, somatostatin ( $1 \mu\text{g}/\text{min}$  per kg; Bachem) was infused to suppress endogenous insulin secretion. A single step of hyperinsulinemia was induced by systemic insulin infusion (regular purified pork; Lilly) from  $t=0$  to 180 min, using one of the following rates:  $0.75 \text{ mU}/\text{min}$  per kg ( $n=41$ ),  $1.15 \text{ mU}/\text{min}$  per kg ( $n=43$ ), or  $1.50 \text{ mU}/\text{min}$  per kg ( $n=6$ ). Euglycemia was maintained by variable rate 50% dextrose infusion, spiked with  $3\text{-}^3\text{H}$ -glucose (specific activity:  $2.2 \mu\text{Ci}/\text{g}$ ). Blood samples were drawn every 10 min from  $t = 10$  to 60 min, every 15 min from  $t = 75$  to 150 min, and every 10 min from 160 to 180 min, and assayed for glucose, insulin, and tracer.
3. **STEP clamp:** After basal blood sampling, glucose was clamped at 3 sequential glycemic levels by exogenous glucose infusion. Target glucose levels were 100 mg/dl ( $t = 0$  to 59 min), 150 mg/dl ( $t = 60$  to 149 min), and 200 mg/dl ( $t = 150$  to 240 min). Samples were assayed for glucose, insulin, and C-peptide.
4. **IVGTT:** After basal blood sampling, glucose ( $0.3 \text{ g}/\text{kg}$ ) was injected at time 0 over 30 seconds. Blood samples were drawn at  $t = 2, 3, 4, 5, 6, 8, 10, 12, 14, 16,$  and 19 min. At  $t = 20$  min, insulin ( $0.03 \text{ U}/\text{kg}$ ; Lilly) was injected, and followed by blood

sampling at  $t = 22, 23, 24, 25, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 140, 160,$  and 180 minutes. Samples were assayed for glucose and insulin.

5. **Fasting Plasma Values:** Fasting blood samples were collected from a peripheral vein on a weekly basis for all animals, and plasma was assayed for glucose, insulin, and free fatty acids (FFA). Mean of two weekly basal samples was used for subsequent analysis.

### Blood Sampling and Assays

Blood samples were collected in lithium and heparin coated tubes containing EDTA (paraoxon, a lipoprotein lipase inhibitor, was an additive for samples used for FFA assay). Samples were centrifuged immediately, and plasma stored at  $-80^{\circ}\text{C}$ . Glucose was assayed on-line in duplicate by the glucose oxidase technique on an automated analyzer (YSI Model 2300), with intra-assay coefficient of variation (CV)  $<1\%$ . Insulin was measured in duplicate by ELISA (Linco Research; St. Charles, MO), with detection limit of 5 pM, and intra- and inter-assay CV of  $2\pm 1\%$  and  $5\pm 1\%$ , respectively. Measurement of  $[3\text{-}^3\text{H}]\text{-glucose}$  was performed after sample deproteinization (13). Plasma FFA were measured by colorimetric assay (Wako; Neuss, Germany). Blood samples for C-peptide assay were collected in lithium and heparin coated tubes containing EDTA, trasylol, and Diprotin A, and quantification was performed using radioimmunoassay (Linco) (7).

### Data Analysis and Calculations

Pooled data from multiple studies were used to examine the relationships between insulin sensitivity and key variables of glucose metabolism and body composition. Specifically, we quantified glucose-stimulated insulin release and insulin clearance, and measured total and regional fat mass. Calculations are outlined below.

1. **Insulin Sensitivity:** The primary measure of whole-body insulin sensitivity calculated for all dogs was obtained from the EGC as previously described (14). Whole-body insulin sensitivity ( $SI_{\text{CLAMP}}$ ) was defined as:

$$SI_{\text{CLAMP}} = \Delta \text{GINF} / (\Delta \text{INS} \times \text{GLU}_{\text{SS}}), \quad (1a)$$

where GINF and INS are the respective increments in glucose infusion rate and insulin measured during exogenous insulin infusion (i.e. steady state minus basal period), and  $\text{GLU}_{\text{SS}}$  is the steady state glucose concentration. After data smoothing (15), rates of glucose uptake ( $R_d$ ) and hepatic glucose output (HGO) were calculated with Steele's equations, modified for use with labeled glucose infusion (16). Peripheral insulin sensitivity ( $SI_{\text{pCLAMP}}$ ) was defined as:

$$SI_{\text{pCLAMP}} = \Delta R_d / (\Delta \text{INS} \times \text{GLU}_{\text{SS}}), \quad (1b)$$

where  $R_d$  is the change in  $R_d$  from basal to steady state. Finally, hepatic insulin sensitivity was calculated as:

$$SI_{HGO_{CLAMP}} = |\Delta HGO / (\Delta INS \times GLU_{SS})|, \quad (1c)$$

where HGO is the observed change in HGO (basal minus steady state). Rates of glucose infusion,  $R_d$ , and HGO were expressed as mg/kg body weight per min for all calculations of clamp-based insulin sensitivity. Given possible variations in body weight among animals, analysis was also performed with data normalized to volume of non-fat tissue obtained from MRIs instead of body weight. Since no difference was observed in statistical strength or conclusions derived from these data,  $SI_{CLAMP}$  results are presented using body weight normalization only. Steady state was defined as the final 30 minutes of the clamp procedure (i.e.  $t = 150$  to 180 min).

Secondary measurement of insulin sensitivity was obtained from IVGTTs performed in a subset of animals ( $n=36$ ). Minimal model analysis was performed to obtain the insulin sensitivity index ( $S_I$ ) using MINMOD Millennium (ver. 6.02; ref. (17)).

2. Glucose-stimulated Insulin Response: Glucose-stimulated insulin response was calculated from STEP clamps ( $n=86$ ) as the slope of the line relating plasma insulin to glucose concentrations, using the mean of final 20 to 30 min from each glycemic period for each variable (i.e. 40-60 min, 120-150 min, and 210-240 min). Incremental area under the curve (AUC) was calculated by the trapezoidal rule.

In a subset of animals ( $n=36$ ), we calculated an additional measure of glucose-stimulated insulin response from the IVGTT. The acute insulin response to glucose ( $AIR_G$ ) was calculated as the incremental insulin area under the curve from 0 to 10 minutes after glucose injection.

3. Insulin Clearance: Three measures of clearance were obtained for this study. The primary measure, available from all animals tested ( $n=90$ ) was the metabolic clearance rate of insulin from the EGC ( $MCR_{CLAMP}$ ), calculated as insulin infusion rate (in  $\mu U/min$  per kg) divided by the mean plasma insulin concentration at steady state (in  $\mu U/ml$ ). Units of  $MCR_{CLAMP}$  are ml/min per kg.

Two additional measures of insulin clearance were obtained from the IVGTT. Fractional insulin clearance was calculated from the decay of plasma insulin after exogenous injection. Insulin from  $t = 20$  to 80 min was fit to the following exponential decay curve using WINSAM software (ver. 3.0.1), as previously described (3):

$$INS = INS_b + INS_{MAX} \times e^{-kT}, \quad [2]$$

where  $INS$ ,  $INS_b$ , and  $INS_{max}$  are the plasma insulin, basal insulin, and peak insulin concentrations (in  $\mu U/ml$ ). Parameter  $k$  represents the fractional clearance rate of insulin ( $FCR_{INS}$ ), expressed in  $min^{-1}$ . Time interval for analysis was chosen to describe full return of insulin to fasting values, while avoiding occasional

fluctuations below basal that can be observed at later time points. It is also assumed that endogenous insulin secretion is suppressed by the exogenous injection (18).

Metabolic clearance rate was also calculated from the IVGTT ( $MCR_{IV}$ ). This parameter, expressed in the same units as the clamp-derived variable (ml/min per kg), is defined as:

$$MCR_{IV} = DOSE_{INS} / AUC_{INS} \quad [3]$$

where  $DOSE_{INS}$  is the dose of injected insulin (0.03 U/kg), and  $AUC_{INS}$  is the above-basal insulin area under the curve from time of injection ( $t = 20$  min) to infinity.

## Statistics

Basic statistics (t-test and ANOVA, with Tukey's post-hoc analysis when overall significance was detected) were performed using MINITAB statistical software (ver. 13.32; State College, PA). Calculated variables were tested for normality using Tukey's ladder test on STATA statistical software (ver. 10), and optimal transformation methods (if required) were determined. When applicable, tests for significance were performed on transformed data. Since data have been pooled from multiple independently performed studies, multiple regression analysis was performed which included controlling for relevant factors which may have differed across studies (i.e. "group" effects), e.g. effects of insulin infusion rate or duration of daily feeding period. Data are reported as mean  $\pm$  SE. When applied to non-normalized data, results are reported as median. Statistical significance was set at  $p < 0.05$ .

## Results

### Fasting Plasma Values

After an overnight fast, baseline variables were within normal ranges in all dogs, and all were of normal glucose tolerance ( $K_G > 1.0 \text{ min}^{-1}$  from IVGTT; ref. (19)). Fasting glucose averaged  $93.5 \pm 0.7$  mg/dl (range: 75 to 106 mg/dl), with minimal variation among animals (CV = 7%; Table 2). Fasting insulin was also normal ( $10.6 \pm 0.6$   $\mu\text{U/ml}$ ), but even at baseline, fasting insulin exhibited much greater relative variability than that observed for glucose (range: 3.1 to 32.4  $\mu\text{U/ml}$ ; CV = 52%). Fasting FFA levels averaged  $0.61 \pm 0.03$  mM, and was not significantly correlated with insulin sensitivity or clearance.

### Body Weight and Adiposity

There was modest variation in body weight amongst animals (range: 20.9 to 37.4 kg; CV = 11%) (Fig. 1B), but this relatively narrow weight range belied great variation in fat distribution patterns. We measured a strikingly wide range of total trunk adiposity (Fig. 1A). Adiposity varied over nearly an 8-fold range, from the leanest animal, with 172  $\text{cm}^3$  of fat mass (7.1% of total trunk tissue) to the most obese animal with 1363  $\text{cm}^3$  total fat, or 39.6% of total tissue (mean:  $601 \pm 25$   $\text{cm}^3$ ,  $21.6 \pm 0.7\%$ ). Variability in total fat was largely driven by subcutaneous adiposity, which varied over a 17.9-fold range between the leanest dog (32  $\text{cm}^3$ , or 1.4% total tissue) to the dog with the greatest subcutaneous adiposity (572  $\text{cm}^3$ , or

18.6% of total tissue). In contrast, visceral adipose mass varied to a significantly lesser degree (~8.6-fold), ranging from 103 to 883 cm<sup>3</sup> (4.2 to 25.6% total trunk tissue, respectively).

### Insulin Sensitivity

Dogs exhibited a wide range of insulin sensitivity under baseline, overnight fasted conditions.  $SI_{CLAMP}$  averaged  $33.5 \pm 1.6$  dl/min per kg per  $\mu\text{U/ml}$  ( $n=90$ ; median = 31.9), but varied nearly 13-fold from most resistant (5.9) to most sensitive (75.9 dl/min per kg per  $\mu\text{U/ml}$ ) animal. This broad range was reflective of a large range in  $SI_{pCLAMP}$  (5.0 to 74.7 dl/min per kg per  $\mu\text{U/ml}$ ; median = 25.0). This range of  $SI_{CLAMP}$  was consistent with another index of total insulin sensitivity, i.e. the minimal model-derived insulin sensitivity index ( $S_I$ ). In the subset of animals undergoing IVGTTs ( $n=36$  of 90 dogs),  $S_I$  averaged  $4.5 \pm 0.4 \times 10^{-4} \text{ min}^{-1}$  per  $\mu\text{U/ml}$ , with a 9-fold range from most resistant to most sensitive ( $1.1$  to  $10.2 \times 10^{-4} \text{ min}^{-1}$  per  $\mu\text{U/ml}$ ). We observed strong correlation between  $SI_{CLAMP}$  and  $S_I$  from the minimal model ( $p=0.001$ ). Consistent with the reported relationship between obesity and insulin resistance, we observed significant inverse associations between  $SI_{CLAMP}$  and total, visceral, and subcutaneous adipose tissue mass, after adjustments for group effects (Table 3;  $p<0.012$ , 0.017, and 0.025, respectively).

### Predictors of Baseline Insulin Sensitivity

In addition to the observed relationship between body composition and insulin sensitivity, we examined whether determinants of circulating insulin are predictive of  $SI_{CLAMP}$  assessed in overnight-fasted animals. Fasting insulin exhibited a significant, negative association with  $SI_{CLAMP}$ , such that resistance was accompanied by fasting hyperinsulinemia ( $p<0.00001$ ; Fig. 2). Since circulating insulin reflects the combined effects of both  $\beta$ -cell secretion and insulin clearance, we explored the impact of each component process to predict  $SI_{CLAMP}$ . The insulin response to hyperglycemia was quantified as the slope relating the glucose-insulin relationship from STEP clamps, as well as the acute insulin response to glucose injection from the IVGTT ( $AIR_G$ ; see METHODS). While these independently obtained measures were highly correlated with each other ( $p=0.0065$ ), neither index was significantly associated with  $SI_{CLAMP}$ . However, the inverse relationship between  $AIR_G$  and insulin sensitivity was detected when the small subset of animals (6 of 36 animals undergoing IVGTTs) with lowest glucose tolerance ( $K_G < 2.0 \text{ min}^{-1}$ ) was excluded from analysis ( $p=0.043$ ).

In contrast, we observed a robust relationship between insulin clearance and clamp-derived  $SI_{CLAMP}$  (Fig. 3A). These variables were positively associated, meaning that decreasing hormone clearance was predictive of Insulin resistance. Of particular interest was the observation that while insulin levels are strongly associated with insulin sensitivity under baseline, overnight-fasted conditions, it is insulin clearance, not secretion that exerts the dominant influence on circulating hormone levels. This finding was independent of experimental procedures, as the observation was confirmed with IVGTT-derived measures of both sensitivity and clearance (Fig. 3B;  $p=0.0016$ ). While  $SI_{CLAMP}$  was influenced by rate of insulin infusion ( $39.78 \pm 2.23$ ,  $28.93 \pm 2.16$ , and  $23.65 \pm 3.54$  dl/min per kg per  $\mu\text{U/ml}$  for infusion rates of 0.75, 1.15, and 1.5 mU/min per kg, respectively), this dose effect did



not alter our primary observation. There was a strong, highly significant linear relationship between clearance and  $SI_{CLAMP}$  at both the 0.75 and 1.15 mU/min per kg infusion rates. [Data from the 1.5 mU/min per kg infusion rate was also highly linear ( $r=0.78$ ), but relationship only approached significance due to small group size ( $p=0.067$ ;  $n=6$  animals).] Slopes defining the clearance-sensitivity relationship were not statistically different across insulin infusion rates ( $p=0.567$ ). Insulin clearance was also strong linked to both peripheral and hepatic insulin sensitivity, which raises the possibility that modulation of clearance could result in improvements in tissue insulin resistance.

## Discussion

The present study was undertaken to examine the metabolic factors which determine insulin sensitivity under baseline, overnight-fasted conditions, in the absence of dietary or pharmacologic intervention. Our data indicate that a primary determinant of baseline insulin sensitivity was the metabolic clearance rate of insulin. This finding was independent of experimental methodology, as the relationship was equally strong whether clamp- or IVGTT-based indices of sensitivity or metabolic clearance rate of insulin are examined. In fact clearance was far more important for determining sensitivity than was  $\beta$ -cell sensitivity to glucose ( $p<0.00001$  for clearance, vs  $p=0.36$  and  $p=0.07$  for AIRg and insulin-glucose slope from STEP clamps, respectively).

In nondiabetic subjects, decrements of insulin sensitivity are associated with increased circulating insulinemia to compensate for reduced insulin action and act to maintain fasting glucose within a narrow range. Compensatory hyperinsulinemia has been thought to result from upregulation of pancreatic  $\beta$ -cells, and elevated insulin secretion is often reported in insulin resistant states (e.g. (4)). However, once secreted, insulin enters the portal vein and hepatic circulation, where at least 50% of the hormone is extracted and metabolized, and the remainder enters the systemic circulation (20). If liver clearance of insulin is reduced, at the same level of insulin secretion, peripheral insulin concentrations will increase. Thus, fasting hyperinsulinemia during insulin resistance can result from increased secretion of insulin, but also from decreased hormone clearance. Indeed, prospective studies of the physiologic response to diet-induced fat feeding reveal that changes in both secretion and clearance contribute to compensatory hyperinsulinemia in dogs (3) and humans (4). The present study demonstrates that insulin clearance may be dominant under baseline, unchallenged conditions as well.

To examine the predictors of insulin sensitivity in a sample of healthy animals, it is necessary that a sufficiently wide range of sensitivity is represented to ensure adequate power to detect key relationships. In this study, the primary measure of insulin sensitivity was obtained from hyperinsulinemic euglycemic clamps. Whole-body insulin sensitivity averaged 33.5 dl/min per kg per  $\mu$ U/ml, but exhibited nearly a 13-fold range from the most insulin resistant animal ( $SI_{CLAMP} = 5.9$ ) to the most sensitive animal ( $SI_{CLAMP} = 75.9$  dl/min per kg per  $\mu$ U/ml). Despite this range, dogs had normal fasting glucose held within a very narrow range (Table 2). These data are consistent with prior reports of a large range of clamp-derived M values across subjects with normal glucose tolerance (2).



Since obesity is often associated with insulin resistance, we examined the relationship between insulin sensitivity and body composition in our study sample. Despite relatively uniform body weight, dogs exhibited a strikingly wide range of adiposity (Fig. 1). Variability was largely driven by subcutaneous fat mass, rather than adiposity within the visceral depot. Despite comparable body weight, subcutaneous fat mass exhibited a striking ~18-fold range across animals, whereas visceral adipose tissue mass varied over half that range (8.6-fold; Table 2). Furthermore, visceral fat mass exhibited a tendency for a more limited capacity for expansion across the wide range of total adiposity, such that fat within the visceral cavity demonstrates a quasi-saturation as total fat deposition increases.

While it is generally believed that obesity is inversely proportional to insulin sensitivity, there is substantial variability in the relationship such that subjects with identical adiposity may exhibit vastly different degrees of sensitivity (21). Despite high degree of variability in both adiposity and insulin sensitivity, we observed a strong negative relationship between these factors in the present study. Total trunk adipose tissue mass was strongly correlated with  $SI_{CLAMP}$  ( $p=0.012$ ), and was similarly strong for discrete adipose tissue mass within the visceral and subcutaneous depots, although these relationships were not apparent in several of the studies used in the present pooled analysis (Table 3). It is also possible that other adipose depots not measured in this study may have stronger influence on insulin sensitivity. It has been proposed that ectopic fat, particularly within the liver, may be the primary driver of obesity-linked insulin resistance (22), and in the absence of increased hepatic fat, elevation of whole body adiposity is not accompanied by insulin resistance (23). This relationship between hepatic fat and insulin sensitivity may also be mediated by insulin clearance, as liver fat is inversely correlated with insulin clearance in diabetic and nondiabetic subjects (24). Such a relationship was not observed in the canine model. Diet-induced obesity induced only modest accumulation of hepatic triglycerides, and hepatic lipid levels were not correlated with direct measures of insulin clearance (25), although in the larger canine cohort described herein, visceral adiposity measured by MRI was negatively associated with clearance. Adipose tissue within the omentum has also been implicated in the development of insulin resistance, and removal of omental fat, representing a small fraction of total fat mass, has marked effects to improve insulin sensitivity in lean animals (26), although this effect was not observed in subjects with morbid obesity ( $BMI > 40 \text{ kg/m}^2$ ) undergoing gastric bypass (27).

What mechanism could explain the relationship between metabolic clearance of insulin and insulin sensitivity? Insulin is cleared from the circulation by many organs, including liver, kidney, adipose tissue, and muscle, but the primary site of hormone clearance is in the liver (28). It is possible that the tissues into which insulin is cleared may be different between the two experimental methods used to quantify the process. During clamps, hyperinsulinemia is sustained for the experimental duration, and insulin-mediated vasodilation (29) may allow greater recruitment of extrahepatic tissues for insulin clearance than may occur during the transient hyperinsulinemia characteristic of the IVGTT. Elevated nocturnal (not fasting) FFA levels have been reported during diet-induced insulin resistance in dogs (7) and may mediate compensatory changes in both  $\beta$ -cell function and insulin clearance. It is plausible that changes in insulin clearance will alter circulating insulin concentrations over the course

of the day, which in turn could affect insulin-sensitive tissues such as skeletal muscle, thus suppressing peripheral insulin action.

There is supportive evidence of each component of this hypothesis. Diminished clearance of insulin increases systemic insulin levels (3,4). Hyperinsulinemia induced by exogenous infusion (30) or end-to-side portocaval shunt (31) impair insulin-stimulated glucose uptake (30). Conversely, subjects with insulinomas who are treated with diazoxide exhibit reduction of hyperinsulinemia secondary to increased insulin clearance, and improved insulin sensitivity (30). The interplay between insulin clearance, circulating insulin, and insulin sensitivity is further supported by studies in transgenic mice with altered expression of CEACAM1, the key regulator of hepatic insulin clearance (32). Mice with liver-specific overexpression of the dominant-negative mutant of CEACAM1 exhibited decreased clearance, with resulting hyperinsulinemia, and animals exhibit marked insulin resistance. Hepatic steatosis has also been reported in mice with liver-specific inactivation of CEACAM1 (33). We have demonstrated prospective changes in CEACAM1 gene expression that track changes in clearance with diet-induced obesity in dogs (34). Insulin clearance is also modulated by insulin degrading enzyme (IDE), a ubiquitous molecule which mediates clearance of insulin and other proteins, including cerebral amyloid  $\beta$ -peptide, from the circulation (35). It is unclear whether IDE activity is altered in states of insulin resistance. Overall, these data are consistent with the hypothesis that insulin clearance is the primary determinant of insulin sensitivity by mediating circulating 24-hr insulin levels, which feed back to regulate peripheral insulin sensitivity. This scenario provides a mechanistic framework by which hepatic insulin clearance is linked to insulin sensitivity.

The question of whether changes in insulin clearance are a determinant of, or a result from, fluctuations in insulin action is difficult to definitively answer from our results. Indeed, we have previously reported a correlation between insulin clearance and sensitivity in a cross-sectional examination of non-diabetic Mexican-American subjects (36), and proposed that changes in clearance contribute to hyperinsulinemic compensation for insulin resistance. However, this prior study was small (n=21), and clearance estimates were indirect and based on suboptimal measures of insulin secretion using nonspecific hormone assays. The present study is large (n=90 animals), and both insulin sensitivity and clearance were measured from independent experimental protocols (IVGTT and clamp) in nondiabetic animals that displayed a broad range of sensitivity. These robust data are consistent with an alternative proposal that changes in clearance precede those of insulin action. Future studies are underway to test this proposal through manipulation of insulin clearance in vivo.

In conclusion, we report that insulin clearance is likely the primary determinant of insulin sensitivity under baseline, overnight-fasted conditions in healthy dogs. Clearance was measured by two independent methodologies from both steady state and dynamic experimental procedures. Among healthy animals of similar body weight and normal fasting glucose, both insulin sensitivity and body adiposity exhibited substantial variability, and were negatively correlated with one another. Of greater magnitude was the strong, positive relationship between insulin clearance and whole body insulin sensitivity. From these results, we propose the hypothesis whereby changes in insulin clearance result in insulin levels that feed back onto insulin action of peripheral and hepatic tissues. Taken together,

these data support a central role for insulin clearance in the regulation of insulin sensitivity. It remains to be determined whether reduction in insulin clearance *per se* could be exploited in human subjects as an approach to enhance insulin sensitivity in insulin resistant individuals at risk for Type 2 diabetes.

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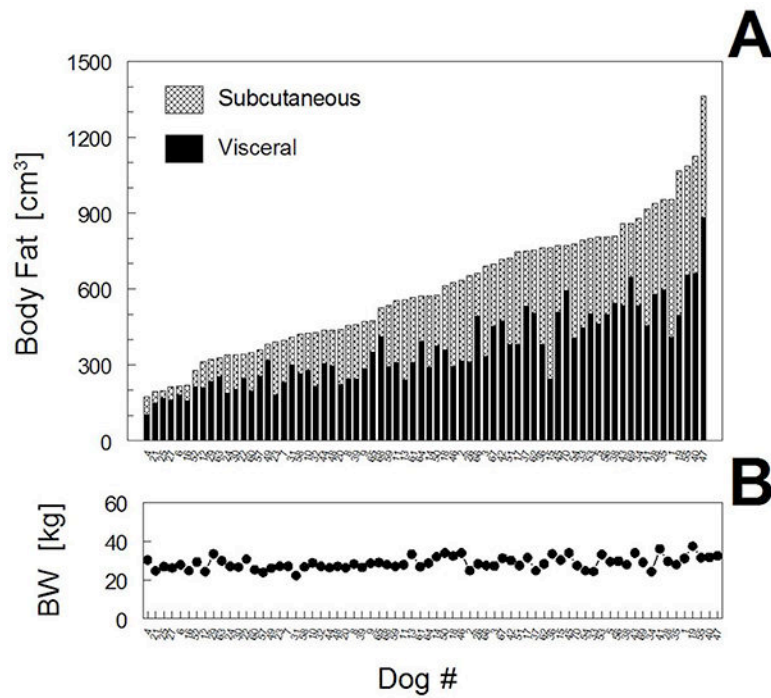
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**What is already known about this subject?**

- Obesity is commonly associated with insulin resistance.
- Insulin resistance is usually accompanied by compensatory hyperinsulinemia in non-diabetic subjects.
- Hyperinsulinemia may result from increased insulin secretion and/or decreased clearance of secreted insulin.

**What does this study add?**

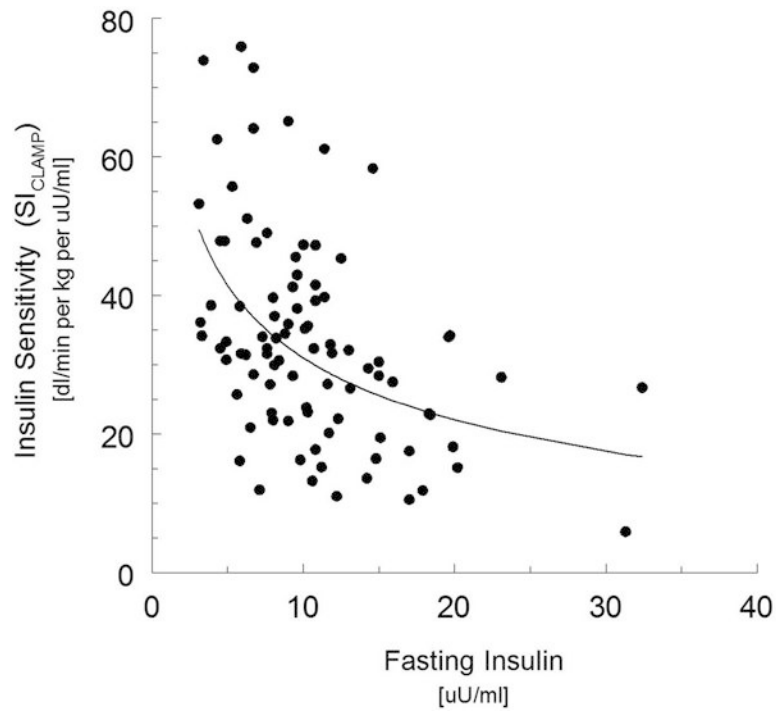
- Wide range of adipose tissue mass (~8-fold range) may exist among subjects of similar body weight.
- Insulin clearance was the dominant factor associated with insulin sensitivity under baseline conditions, whether sensitivity was calculated from euglycemic clamp or intravenous glucose tolerance test.
- Data support the hypothesis whereby changes in insulin clearance result in insulin levels that feed back onto insulin action of peripheral and hepatic tissues.



**Figure 1. Variability in adiposity and body weight in normal dogs**

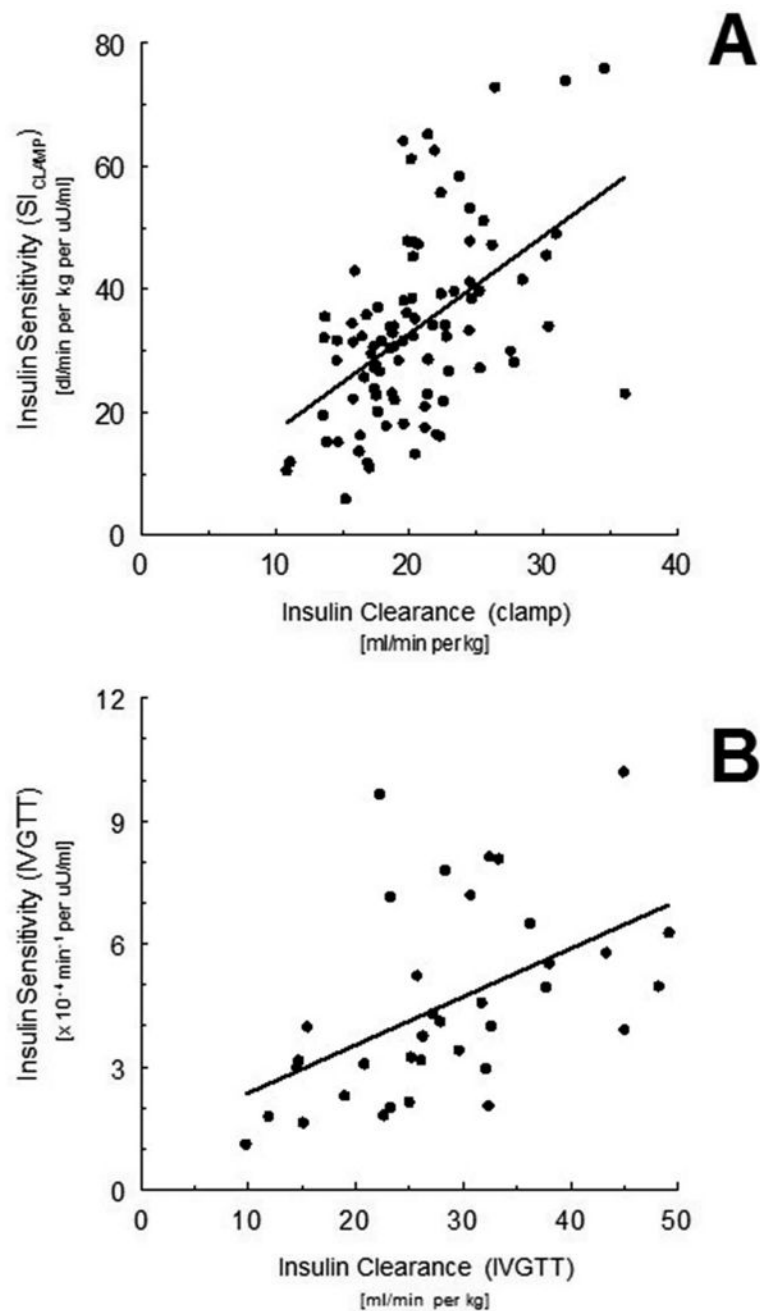
(A) dogs ranked by total adiposity (height of stacked bars), with respective visceral (solid) and subcutaneous (hatched) fat mass. (B) Body weight for dogs ranked by total fat. Note that variability of total and subcutaneous fat greatly exceeds that of body weight.





**Figure 2. Correlation between clamp-based insulin sensitivity and fasting insulin under baseline conditions**

Fasting insulin was inversely correlated with  $SI_{CLAMP}$ , which confirms that insulinemia may well contribute to the prevailing degree of insulin sensitivity, even in the absence of dietary or pharmacologic perturbation.



**Figure 3. Strong correlation between insulin sensitivity and metabolic clearance rate of insulin using indices derived from (A) euglycemic hyperinsulinemic clamp and (B) IVGTT**  
Regardless of experimental methodology, a lower degree of insulin sensitivity was associated with low insulin clearance, which contributes to the hyperinsulinemia required as compensation for insulin resistance.

**Table 1**

Outline of specific procedures performed in healthy animals.

Reference	Total # Dogs	Procedures Performed (# dogs)					
		MRI	EGC	STEP Clamp	IVGTT		
(5)	26	26	26	26	26	----	----
(6)	6	6	6	6	6	----	----
(7)	8	8	8	4	4	----	----
(8-10)	21	21	21	21	21	8	8
(11)	17	17	17	17	17	17	17
(12)	12	12	12	12	12	11	11
Total	90	90	90	90	86	36	36

Table 2

Variability of key metabolic variables under baseline conditions.

Parameter	n	Mean±SE	Minimum	Maximum	Fold Range
<i>Fasting Plasma Values</i>					
Glucose	90	93.5±0.7	75.4	106.0	1.4
Insulin	90	10.6±0.6	3.1	32.4	10.5
FFA	90	0.61±0.03	0.24	1.45	6.0
<i>Body Composition</i>					
BW	90	28.7±0.3	20.9	37.4	1.8
Total Fat	90	600±25	172	1363	7.9
Visc Fat	90	368±15	103	883	8.6
SQ Fat	90	233±13	32	572	17.9
<i>Insulin Sensitivity</i>					
SI <sub>CLAMP</sub>	90	33.5±1.6	5.9	75.9	12.9
SI <sub>IVGTT</sub>	36	4.5±0.4	1.1	10.2	9.1
<i>Glucose-Stimulated Insulin Response</i>					
AIR <sub>GLU</sub>	36	609±38	217	1213	5.6
SLOPE <sub>INS</sub>	86	1.14±0.13	0.17	8.03	46.1
<i>Insulin Clearance</i>					
MCR <sub>CLAMP</sub>	90	20.5±0.5	10.8	36.1	3.3
FCR <sub>IV</sub>	36	0.50±0.05	0.15	1.72	11.5
MCR <sub>IV</sub>	36	28.4±1.7	9.8	49.1	5.0

**Table 3**Regression coefficients describing overall and group-dependent effects of adiposity on SI<sub>CLAMP</sub>.

Study Group	Coefficient	SE	p-value
<b>Total Fat: Overall Effect, <math>p=0.0003</math> (<math>r=0.51</math>)</b>			
Reference Group	5.983	0.360	0.000
Group 2	0.098	0.365	0.789
Group 3	-0.567	0.524	0.282
Group 4	0.966	0.361	0.009
Group 5	1.641	0.471	0.001
Group 6	1.259	0.407	0.003
Effect of Total Fat *	-0.0014	0.0006	0.012
<b>Visceral Fat: Overall Effect, <math>p=0.0004</math> (<math>r=0.50</math>)</b>			
Reference Group	6.801	0.671	0.000
Group 2	0.303	0.392	0.441
Group 3	-0.449	0.525	0.395
Group 4	1.102	0.389	0.006
Group 5	1.766	0.482	0.000
Group 6	1.505	0.436	0.001
Effect of Visceral Fat *	-0.0955	0.0391	0.017
<b>Subcutaneous Fat: Overall Effect, <math>p=0.0006</math> (<math>r=0.49</math>)</b>			
Reference Group	6.277	0.502	0.000
Group 2	-0.094	0.363	0.797
Group 3	-0.651	0.532	0.225
Group 4	0.768	0.346	0.029
Group 5	1.545	0.471	0.002
Group 6	1.044	0.407	0.012
Effect of Subcutaneous Fat *	-0.0699	0.0307	0.025

Pooled data set was comprised of individual studies performed by authors. Statistical analysis was performed to determine overall effect or effects of each individual study group s, defined as follows: Reference group (5), Group 2 (11), Group 3 (6), Group 4 (8,9), Group 5 (7), Group 6 (12). Statistics were not appreciably altered by choice of reference group.

\* Measures of SI<sub>CLAMP</sub> and adiposity were normalized by square root transformation prior to statistical analysis (see METHODS).