Variability of directly measured first-pass hepatic insulin extraction and its association with insulin sensitivity and plasma insulin

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Running title: Distribution of First-pass Hepatic Extraction

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Abstract
While the β-cells secrete insulin, it is the liver with its first-pass insulin extraction (FPE) that regulates the amount of insulin allowed into circulation for action on target tissues. The metabolic clearance rate of insulin, of which FPE is the dominant component, is reported to be a major determinant of insulin sensitivity (SI). We studied the intricate relationship between FPE, SI and fasting insulin. We used a direct method of measuring FPE, the paired portal/peripheral infusion protocol (PPII) where insulin is infused step-wise, either via the portal vein or a peripheral vein in healthy young dogs (n =12). FPE is calculated as the difference in clearance rates (slope of infusion rate vs. steady insulin plot) between the paired experiments. Significant correlations were found between FPE vs. clamp assessed SI (r_s = 0.74); FPE vs. fasting insulin (r_s = -0.64) and SI vs. fasting insulin (r_s = - 0.67). Also, we found a wide variance in FPE (22.4 - 77.2%; mean ± SD of 50.4 ± 19.1%) which is reflected in the variability of plasma insulin (48.1 ± 30.9pM) and SI (9.4 ± 5.8 x10^4 dL·kg^{-1}·min^{-1}·pM^{-1}). FPE could be the nexus of regulation of both plasma insulin and SI.

Plasma insulin is determined by pancreatic β cell secretion and metabolic clearance (MCR) of the hormone. MCR is the aggregate catabolism of insulin by all insulin-sensitive tissues of which the liver is dominant, extracting up to ~80% of secreted insulin (1). Insulin is secreted into the portal vein. A varying amount of the insulin is extracted by the liver during the passage of portal insulin (FPE, first-pass extraction) which controls the amount of secreted insulin to access the periphery for action on intended extrahepatic tissues. Importantly, the hyperinsulinemic
compensation during the induction of insulin resistance is the combination of both increased insulin secretion and reduced FPE (2, 3). During persistent insulin resistance, however, it is the reduction in FPE that primarily sustains the hyperinsulinemic compensation (2, 3), yet less attention has been focused on FPE compared to β cell function in the determination of plasma insulin. Rodent studies have shown that inhibition of hepatic insulin extraction causes hyperinsulinemia, insulin resistance and attendant disorders (4, 5). African Americans, for example, have reduced FPE (6), elevated plasma insulin (7), low insulin sensitivity (SI) (7), and a high risk of metabolic diseases compared to Caucasians (8, 9). These reports support the essentiality of FPE in insulin resistance and associated metabolic diseases, and suggest the need for more attention to be focused on FPE. Low FPE could be a major risk factor for insulin resistance. Identification of high-risk persons (low FPE) with appropriate interventions could be vital to controlling insulin resistance and its associated comorbidities. Due to the practical difficulty of direct measurement of FPE, as deep-seated vessels need to be cannulated, clinical studies often use surrogate methods. Using a large animal model, the canine, which enables us to measure hepatic insulin extraction directly, we investigate the relationship between FPE, SI and plasma insulin in a sample of healthy animals.

**Research Design**

**Animal Care**

Under the oversight of Cedars Sinai Medical Center’s Institutional Animal Care and Use Committee, the animals were housed under 12hr:12hr light and dark cycle in a temperature controlled vivarium where they were inspected daily by board-certified veterinarians. The dogs were given a standard diet comprising one can of Purina Proplan Classic Puppy Chow (10% protein, 7% fat, 1.5% fiber and 76% moisture; Nestle Purina PetCare Company, St Louis, MO)
and 825g of dry chow (2.9% fiber, 27.7% protein, 29.9 % fat and 42.4 % carbohydrate; LabDiet, PMI Nutrition Int’l, Brentwood, MO) for a total of 3,576 calories/day of which 39.2% from carbohydrate, 32.5% from fat and 28.3% from protein. They always had access to water.

**Surgery**

Twelve male mongrel dogs, ~1yr old underwent laparotomic surgery and 7” French catheters were inserted approximately 3.5cm distal from the porta-hepatis and secured in place with 5.0 prolene suture. The catheter was then tunneled subcutaneously and connected to a planted vascular access port (VAP) at the iliocostalis lumborum muscle. Liver biopsies were also taken and snap-frozen for molecular assessments. The abdominal cavity was sutured using 2.0 monocryl sutures. The dogs were allowed 2-3 weeks to recover and achieve steady body weight. The VAP and catheter were kept patent by locking with 2.0 mL Taurolidine-Citrate catheter solution (Access Technologies, Skokie, IL). Experiments were carried out after an overnight fast and before each study body temperature and hematocrit were checked.

**Methods**

Three randomized experiments, listed below, were performed on each of the twelve dogs in the conscious state. The experiments were separated by at least 3 days. The portal and peripheral insulin infusion protocols were paired experiments and as such were performed consecutively, separated by the recovery days.

I. Hyperinsulinemic-Euglycemic Clamp (EGC)

II. Portal Insulin Infusion Protocol

III. Peripheral Insulin Infusion Protocol
Hyperinsulinemic Euglycemic clamp (EGC)

At t = -120min, a primed infusion (25 uCi + 0.25 uCi/min) of [3-$^3$H]-glucose (PerkinElmer, Waltham, MA) to estimate glucose turnover was started and continued throughout the experiment. After 90min of tracer equilibration, 4 basal samples were taken, 10min apart from t = -30min. After the last basal sample, at t = 0min, a continuous peripheral infusion of 4.5pmol/kg/min of porcine insulin (Eli Lilly, Indianapolis, IN) to sustain hyperinsulinemia and 1.0µg/kg/min of somatostatin (Bachem, California, Torrance, CA) to inhibit insulin and glucagon secretion were started and maintained until the end of the experiment. To maintain euglycemia, a variable rate infusion of 50% (454mg/ml) dextrose (GINF) mixed with [3-$^3$H]-glucose for a specific activity of 2.0 uCi/g was adjusted as necessary. Blood samples were taken every 10min from t = 0 - 60min, every 15min from t = 60 -120min, and every 10min from t = 120 -180min. From 150 -180min was considered the steady state.

Paired peripheral/portal insulin infusion protocol (PPII)

After 3 basal samples were taken at the times -110, -100, and -91min, 1.0ug/kg/min somatostatin was started at t = -90min via a saphenous vein and throughout the experiment to inhibit insulin secretion during either the portal or peripheral insulin infusion protocol. Beginning at t = 0min, replacement glucagon at 1.3ng/kg/min was infused into the portal vein and continued throughout the experiment during both portal and peripheral insulin infusion protocols. Plasma glucose was clamped at the measured basal concentration during the experiments by a variable rate glucose infusion through a saphenous vein.

Peripheral Insulin Infusion during Clamp
Starting at \( t = 0 \text{min} \), insulin was infused at three successive incremental rates: 1.5, 3.0 and 4.5pmol/kg/min through a peripheral vein. Each infusion rate lasted for 90min of which the last 30min is considered as the steady state. Blood samples were taken every 10min from another peripheral vein for the assays.

**Portal Insulin Infusion during Clamp**

Through the portal vein, insulin was infused at three successive incremental rates (2x the rate of peripheral infusion); 3.0, 6.0 and 9.0pmol/kg/min. Each infusion rate spanned 90min and the last 30min of each rate was considered the steady state. Blood samples were taken every 10min from a peripheral vein. Twice the insulin infusion rates of the peripheral clamp experiments were used for the portal protocol, with the aim of achieving matching circulating plasma insulin between the two experiments, on the assumption that the liver extracts about 50% of portal insulin (10). The infusion rates used, cover the physiological ranges of plasma insulin, from fasting to post prandial.

**Sample collection, glucose, insulin and tracer assays**

One mL of blood was taken at each time point from a peripheral vein into chilled 1.5mL Eppendorf tubes coated with lithium fluoride, heparin and 1ug/50ul EDTA. Collected samples were immediately centrifuged, plasma aliquoted, and glucose concentrations measured with YSI 2700 autoanalyzer (Yellow Springs Instruments, Yellow Springs, OH). The rest of the plasma samples were stored at -20°F until ready for insulin measurements and \([3^-3\text{H}]-\)glucose tracer assay in the case of the EGC. A sandwich ELISA kit designed for porcine and canine insulin (ALPCO, Salem, NH; catalog # 80-INSPO-E01) was used to assay the insulin. The ELISA assay has an identical sensitivity to porcine and canine insulin. The intra and inter-assay CV of insulin was 2.3 ± 0.3% and 2.9 ±1.3 % respectively. Samples were processed on ice. Averages of
baseline insulin measurements of each dog before the experiments were considered as the fasting insulin. The plasma samples from the EGC were processed according to Ader and Bergman (11) and the specific activity of the \([3^{-3}\text{H}]-\text{glucose}\) tracer assayed by a liquid scintillation counter (LS 6000SC, Beckman Instruments Inc., Fullerton, CA).

**Liver function assays**

The liver function panels were assayed from fasting blood samples by Antech Diagnostic (Irvine, CA), a veterinary laboratory service provider.

**Total RNA Isolation and Gene Expression Assays**

TRI Reagent Kit (Molecular Research Center, Cincinnati, OH) was used for the extraction of the total RNA from the liver biopsies according to the accompanying protocol. One microgram of the total RNA was reversed transcribed by Invitrogen’s SuperScript First-Strand Synthesis System for Real Time-Polymerase Chain Reaction kit (ThermoFisher Scientific/Invitrogen, Carlsbad, CA) to first strand cDNA. Predesigned TaqMan real-time PCR assays (Insulin receptor (INSR), Cf02647625_m1; Insulin Degrading enzyme (IDE), Cf02634270_m1; Carcinoembryonic Antigen Related Cell Adhesion Molecule 1 (CEACAM1), Cf03054097_m1 and 18s rRNA, Hs99999901_s1: ThermoFisher Scientific/Applied Biosystems, Foster City, CA) with Roche’s Light-Cycler 4.8 instrument (Roche Applied Science, Indianapolis, IN) were used to assess the gene expressions. Data were analyzed by the relative quantification method using 18s rRNA gene as the internal control for normalization.

**Calculations**

**Insulin Sensitivity (SI) from the Hyperinsulinemic Euglycemic Clamp**
SI = \left( \frac{\Delta GINF}{\Delta Ins \cdot Glc} \right) \quad (1)

\Delta GINF = \text{glucose infusion rate at steady state normalized by body weight } - \text{glucose infusion rate at basal normalized by body weight}

\Delta Ins = \text{plasma insulin concentration at steady state } - \text{plasma insulin at basal}

Glc = \text{glucose concentration at steady state}

From the infused tracer during the EGC, we also calculated hepatic insulin sensitivity, SiHGO, and peripheral insulin sensitivity, SiP, according to the respective equations below (12, 13).

\[
SiHGO = \left| \frac{\Delta HGO}{\Delta Ins \cdot Glc} \right| \quad (2)
\]

\[
SiP = \frac{\Delta Rd}{\Delta Ins \cdot Glc} \quad (3)
\]

Hepatic glucose production, HGO, and peripheral glucose uptake, Rd, were calculated from the modified Steele’s equation (14), where \(\Delta HGO\) (suppression of glucose production) and \(\Delta Rd\) (increasing peripheral glucose uptake) are the respective changes in HGO and Rd from basal to steady state during the EGC.

**First-pass Hepatic Insulin Extraction (FPE) from the Paired Portal/Peripheral Insulin Infusion Protocol**

From the isotopic dilution principle (1):

\[
\text{Insulin clearance rate, } CL (\text{mL/kg/min}) = \frac{\text{insulin infusion rate (pmol/kg/min)}}{\text{Steady state plasma insulin conc. (pmol/L)}} \quad (4a)
\]
Assuming linear insulin kinetics within the concentration studied, insulin clearance rate (CL), can thus be calculated as the inverse of the slope, m of the least squares regression line between insulin infusion rate (pmol/kg/min) and steady state plasma insulin concentration (pmol/L) (3, 11). Hence CL can be expressed as;

\[
CL \text{ (ml/kg/min)} = \frac{1}{m \text{ (kg*min/ml)}} \tag{4b}
\]

First–pass Hepatic Insulin Clearance Rate (ml/kg/min) = CLpo – CLpe \tag{5}

Where CLpo and CLpe are insulin clearance rates during the portal infusion and peripheral infusion protocols respectively.

First – pass Hepatic Extraction, FPE (%) = \left( \frac{CLpo – CLpe}{CLpo} \right) \times 100 \tag{6}

Substituting equation (4b) into (6) gives equation (7) below;

\[
FPE \text{ (%)} = \left( 1 - \left( \frac{mpo}{mpe} \right) \right) \times 100 \tag{7}
\]

Where mpo and mpe are the respective slopes of portal infusion rate vs. steady state plasma insulin and peripheral infusion rate vs. corresponding steady state plasma insulin.

In analyzing the distribution of FPE, we include the baseline assessments of six dogs from a published study (3), which underwent the same experimental protocol for FPE measurements as the current twelve, in order to increase the sample size.
Statistics

Spearman’s rank order correlation, $r_s$, is used for the association analysis. A p-value less than 0.05 is the set level of significance. All data are reported as mean ± SEM unless stated otherwise.

Results

The average body weight of the 12 dogs was 27.5 ± 1.0kg. Mean fasting insulin was 48.1 ± 8.9pM, spanning from 23.3 - 118.4pM, a 5.1fold range, and fasting glucose was 95.3 ± 1.4mg/dL, indicating no glucose dysregulation. Two dogs had high fasting insulins of 104.4 and 118.4pM respectively. For one of those 2 dogs, the fasting insulin at 3 different days, each with ≥ 3 samples were 108.9, 126.1 and 120.2pM while that of the other dog were 113, 101.6 and 98.7pM. The consistent high insulin levels indicate that they were not spurious measurements.

The mean basal HGO was 2.0 ± 0.1mg·kg⁻¹·min⁻¹, from 1.2 - 2.5mg·kg⁻¹·min⁻¹, a 2.1fold range. Mean SI was 9.4 ± 1.7 x10⁴ dL·kg⁻¹·min⁻¹·pM⁻¹, from 3.0 - 26.2 x10⁴ dL·kg⁻¹·min⁻¹·pM⁻¹, an 8.8fold range. The mean SiHGO was 2.7 ± 0.6 x10⁴ dL·kg⁻¹·min⁻¹·pM⁻¹, from 1.1 - 8.7 x10⁴ dL·kg⁻¹·min⁻¹·pM⁻¹, a 7.7fold range while the mean SiP was 6.7 ± 1.2 x10⁴ dL·kg⁻¹·min⁻¹·pM⁻¹ from 1.7 - 18.0 x10⁴ dL·kg⁻¹·min⁻¹·pM⁻¹, a 10.6fold range. FPE spanned from 22.4 - 77.2%, a 3.5fold range (Table 1). Liver function panels were normal (Table 2). Considering the wide range of FPE realized, we added the baseline data of 6 dogs from a previously published study [3] to increase the sample size as we assessed the variability of FPE in normal dogs. For the 18 dogs analyzed for the variability of FPE, mean FPE was 53.4% with a standard deviation of 16.5% (Fig. 6).
Correlations

We found significant correlations between FPE vs. SI ($r_s = 0.74$, $p = 0.006$; Fig. 2A), FPE vs. fasting plasma insulin ($r_s = -0.64$, $p = 0.03$; Fig. 2B) and SI vs. fasting insulin ($r_s = -0.67$, $p = 0.02$; Fig. 2C). We then omitted the data of the two dogs with outlier high fasting insulin and reassessed the correlations between FPE, SI and fasting insulin. The correlations remained significant and stronger even after omitting the data of those two dogs; FPE vs. SI ($r_s = 0.82$, $p = 0.004$), FPE vs. fasting insulin ($r_s = -0.78$, $p = 0.008$), SI vs. fasting insulin ($r_s = -0.78$, $p = 0.008$). We further analyzed the correlations between FPE and the components of SI, SiHGO and SiP using the data of all 12 animals. We found a significant correlation between FPE vs. SiP ($r_s = 0.80$, $p = 0.002$; Fig. 3A) and a similar relationship between FPE vs. $\Delta R_d$, from which SiP was defined ($r_s = 0.75$, $p = 0.005$). However, we found an insignificant correlation between FPE and SiHGO ($r_s = 0.31$, $p = 0.33$; Fig. 3B) and a corresponding relationship between FPE vs. $|\Delta HGO|$, from which SiHGO was calculated ($r_s = 0.34$, $p = 0.28$).

IDE and CEACAM1 are key proteins involved in hepatic insulin catabolism, after insulin is bound to its receptors (4, 10, 15). We assessed how the expressions of these genes relative to 18s rRNA (arbitrary unit) are associated with FPE. We found a significant association between INSR vs. FPE ($r_s = 0.67$, $p = 0.02$; Fig. 4A). However, IDE and CEACAM1 did not significantly correlate with FPE; CEACAM1 vs. FPE ($r_s = 0.47$, $p = 0.12$; Fig. 4B) and IDE vs. FPE ($r_s = 0.31$, $p = 0.33$; Fig. 4C). We did not also find a significant correlation between HGO vs. INSR ($r_s = 0.21$, $p = 0.51$; Fig. 5A). The correlation between FPE and basal HGO was 0.50 with a p-value of 0.10 (Fig. 5B).

Additionally, there was no significant correlation between FPE vs. fasting plasma alanine aminotransferase (ALT) or aspartate aminotransferase (AST). These aminotransferases are used
as measures of liver’s health (16). Similarly, no correlation was realized between FPE vs. fasting albumin and total bilirubin, clinical surrogates of hepatic function (16).

**Discussion**

Plasma insulin concentrations are regularly used as the gauge for SI (2, 17). While insulin is secreted by the pancreas, it is the liver that determines how much insulin is allowed into the general circulation for its eventual action on target tissues. Studies have suggested a cross-talk between insulin secretion and hepatic insulin extraction in ensuring adequate insulinemia to maintain normal glucose tolerance (3, 18). It is acknowledged that during insulin resistance, which can be a regular physiological change, as in pregnancy (19), puberty (20), old age (21), or a pathophysiological condition such as obesity (2), a combination of increased secretion and reduced hepatic extraction result in hyperinsulinemia to compensate for the reduced insulin action (2, 3).

However, it is also postulated that decreased hepatic insulin clearance causes hyperinsulinemia which in turn causes the insulin resistance (4, 22). As such, decreased hepatic insulin extraction is purported to be the cause of insulin resistance rather than a reaction thereof (22). Thus, it is essential that considerable attention is focused on FPE, the “gatekeeper” of plasma insulin, as much as the β-cell function in the determination of the systemic levels of the hormone and SI. The significant correlations between FPE, SI and fasting insulin (Fig. 2) found in this cross-sectional study, while they do not explain whether reduced FPE is the cause or consequence of insulin resistance, they do suggest a role of FPE as the nexus for the regulation of plasma insulin level and SI.
FPE controls plasma insulin. Metabolic clearance of insulin, which includes FPE is a strong determinant of SI (12). Insulin extraction and insulin action are both mediated by identical receptors, which could possibly explain the correlation between FPE and SI. SiHGO and SiP account for the whole-body insulin sensitivity, SI. It is apparent that the relationship between FPE and SI ($r_s = 0.74$, $p = 0.006$; Fig. 2A), is driven predominately by SiP and not so much by SiHGO, due to the strong association between FPE vs. SiP (Fig. 3A) and the poor correlation between FPE and SiHGO (Fig. 3B). In fact during EGC, insulin’s action at the peripheral, in stimulating glucose uptake into skeletal muscles and adipose tissue is expansive than its action on the liver (11). The dissociation of FPE and HGO/SiHGO could stem from the differential expressions and binding affinities of the alternatively spliced isoforms of the insulin receptor, IR-A and IR-B (23). IR-A binds to insulin with a higher affinity, about twice that of IR-B, however, in the liver IR-A has a lower expression level than the IR-B (23). Though the functional significance of the differences in binding affinities and expression levels of the isoforms is not completely clear, IR-A is reported to bind to CEACAM1 to mediate hepatic insulin extraction (15). Additionally, HGO is both directly and indirectly controlled by insulin (11, 24). Indirectly, secreted insulin allowed into systemic circulation after FPE regulation, act to change signals elsewhere in the body (e.g., Central Nervous System, adipose tissue’s lipolysis, glucagon secretion) which subsequently inhibit HGO (25, 26). Thus, if the indirect control is dominant, there could be a dissociation between FPE and HGO/SiHGO. The strong correlation between FPE vs. SiP ($r_s = 0.80$, $p = 0.002$; Fig. 3A) and the dissociation between FPE vs. HGO/SiHGO, together, support the dominance of indirect control of HGO by insulin. Additionally, the lack of significant correlation between basal HGO and relative expression of hepatic INSR at basal does not indicate a dominant role for insulin’s direct effect on HGO.
The metabolic clearance of insulin is reported to be the most formidable determinant of SI, much more so than fasting insulin in overnight fasted dogs (12). The strong correlation between FPE and SI ($r_s = 0.74$; $p = 0.006$) compared to fasting insulin vs. SI ($r_s = -0.67$; $p = 0.02$) reinforces the important role of insulin clearance in whole body insulin sensitivity. Furthermore, the high variability of FPE ($50.4 \pm 19.1\%$; mean $\pm$ SD; Table 1) seems to be reflected in the SI ($9.4 \pm 5.8 \times 10^4$ dL$^{-1}$kg$^{-1}$min$^{-1}$pM$^{-1}$; mean $\pm$ SD) and fasting insulin ($48.1 \pm 30.9$ pM; mean $\pm$ SD).

**Wide Interindividual Variation in FPE**

Of interest, is the wide distribution of FPE in a cohort of normal animals. The expansive intrapopulation differences, as evidenced in the FPE (Fig. 6), could be due to genetic factors (27). Genetic association studies identified chromosomal regions linked to SI and insulin clearance (28). From studies in Mexican Americans, Goodarzi et al. (29) reported that MCR of insulin is highly heritable, and a follow-up study identified 18 associated SNPs (30). Such heritable allelic genes with varying degrees of penetrance possibly underscore the high variability in insulin clearance. FPE seems also to be a highly regulated process. After nutrient ingestion (31, 32) and during marginal changes in body weight, even within normal BMI (17), FPE undergoes dynamic changes to accommodate the insulin needs of the body; an indication of a tightly controlled mechanism. Perhaps low FPE signifies dysregulation of insulin clearance which actuates hyperinsulinemia and insulin resistance.

CEACAM1 is involved in the internalization of the bound insulin on the hepatocytes (4, 15), and in the endosome, IDE initiates the catabolism of insulin (10). The absence of significant correlation between IDE, CEACAM1 and FPE (Fig. 4) in this study despite their previously reported roles in hepatic insulin metabolism might be due to the lack of statistical power. IDE,
while the principal catabolic enzyme of insulin (10), is also upregulated by insulin (33). Perhaps
the increased expression levels of the gene during the hours-long insulin infusion of the PPII
protocol is what might be significantly correlated with the FPE, and not the basal expression
assayed in this study. The significant association between FPE, estimated through
hyperinsulinemic conditions and the relative expression of INSR at basal (Fig. 4A), indicates the
critical role of the insulin receptors in hepatic insulin extraction. Unlike insulin’s control of HGO
where the receptors are dispensable, hepatic insulin extraction requires the insulin receptors (24,
34).

The wide variation of FPE recorded in this study has also been reported in other studies using
different methods. From direct sampling of the portal vein, hepatic artery, and hepatic vein with
plasma flow rate measurements, Kadan et al. (35) reported a basal range of -20 to 90% (40 ±
11%; mean ± SD) in anesthetized dogs; and in conscious dogs, Jaspan et al. (36) noted a sample
mean of 31% with a standard deviation (SD) of 11%. In humans, using C-peptide deconvolution
derived insulin secretion and plasma insulin kinetics, Polonsky et al. (37) reported a mean of 53%
at basal with an SD of 14% in 14 healthy weight subjects while Meier et al. (38) reported 78 ±
10% (mean ± SD) in 5 subjects. C-peptide is co-secreted with insulin, but unlike insulin, it is not
significantly extracted by the liver; thus the ratio of C-peptide to insulin is used as an index of
FPE (39). Different clinical studies with hepatic vein sampling and peripherally infused insulin
measured 40-85% single pass splanchnic insulin extraction (39-41). Splanchnic insulin
extraction encompasses insulin uptake by hepatic and extrahepatic visceral tissues, but the liver
is the primary catabolic organ of the hormone (42).

**Methods of measuring FPE**
The distinct advantage of the PPII method is that sampling is done only from a peripheral vein and not from deep-seated vessels (portal vein, hepatic artery and hepatic vein) which are difficult to sample from. The unique challenge to the optimal representative sampling of portal blood due to streaming, the pulsatility of β-cell discharge and sampling error (43-45) resulted in disparate negative extraction values in some studies (35, 45). Streaming due to the low velocity of portal blood could result in inadequate mixing of blood from the different vessels emptied into the portal vein (46-48). Sampling error (43-45) occurs due to the difficulty in drawing blood from these deep-seated vessels, which possibly confounds the measurement of hepatic extraction. Furthermore, the blood flow rate through a catheterized hepatic vein is altered compared to the other hepatic veins (49). Thus, estimating hepatic extraction fraction from one catheterized hepatic vein might not reflect entirely the composite liver uptake of insulin.

Furthermore, C-peptide based methods of measuring FPE assume negligible C-peptide extraction by the liver. However, 3 of the 13 dogs used to accredit the insignificant average C-peptide uptake by the liver had appreciable hepatic C-peptide extraction values of 21 to 35 % and another had a negative extraction percentage (45). Thus, in about a quarter of the studied dogs (45), C-peptide/insulin ratio could not be used as an accurate surrogate for FPE.

Nevertheless, PPII is limited by the reliance on constant infusion rates and steady-state plasma insulin to calculate the FPE. Some studies have reported that the pulsatile secretion of insulin determines its fractional hepatic extraction (38, 50). Also, PPII assumes that the plasma flow rate is the same for both experiments. Though our sample mean of 53% is consistent with historic averages from endogenous pulsatile secretion (45), future studies will analyze the correlation between FPE from PPII and that of direct sampling from the hepatic artery, portal and hepatic veins. We also plan for a longitudinal study to ascertain if animals with low FPE do
develop insulin resistance and its attendant diseases relatively quickly compared to those with high or average FPE when subjected to high-fat diet challenge. A limitation to this study is the inability to assess the two isoforms of the insulin receptor and analyze how they relate with HGO and FPE.

In summary, using a direct method of estimating FPE, the PPII we found significant correlations between FPE, fasting insulin and SI. Perhaps FPE regulates not only plasma insulin but also SI. Also, we found a wide interindividual variation of FPE in normal healthy dogs suggesting differential regulation of FPE. Possibly, poorly regulated low FPE might be a risk for hyperinsulinemia and insulin resistance.

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**Authors Contributions.** I.A.B. contributed to the study design, performed experiments, analyzed data and wrote the manuscript. R.L.P. performed experiments and edited the manuscript. S.P.K. conceived the study, designed the study, wrote the protocol and analyzed data. O.O.W. performed surgeries and edited the manuscript. C.K.M. reviewed the manuscript. M.A.B. performed surgeries. M.K. performed the molecular assay and edited the manuscript. RNB contributed to the study design and reviewed the manuscript. R.N.B. is the guarantor of this work, and, as such, had full access to all the data in the study, and takes responsibility for the integrity of data and accuracy of the data analysis.

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## List of Tables

Table 1. First-pass hepatic insulin extraction, fasting insulin, fasting glucose production, whole-body insulin sensitivity, hepatic insulin sensitivity and peripheral insulin sensitivity of respective dogs.

| Dog No. | FPE (%) | Fasting Insulin (pm) | Fasting HGO (mg·kg⁻¹·min⁻¹) | SI x10⁻⁴ (dL·kg⁻¹·min⁻¹·pM⁻¹) | SiP x10⁻⁴ (dL·kg⁻¹·min⁻¹·pM⁻¹) | SiHGO x10⁻⁴ (dL·kg⁻¹·min⁻¹·pM⁻¹) |
|---------|---------|----------------------|-----------------------------|-----------------------------|-------------------------------|---------------------------------|
| 1       | 22.37   | 57.0                 | 1.22                        | 6.95                        | 4.02                          | 2.43                            |
| 2       | 24.33   | 38.85                | 1.44                        | 2.99                        | 1.70                          | 1.14                            |
| 3       | 31.82   | 39.13                | 1.87                        | 7.19                        | 5.59                          | 1.60                            |
| 4       | 39.24   | 41.65                | 2.19                        | 9.40                        | 6.85                          | 2.16                            |
| 5       | 42.49   | 104.43               | 2.29                        | 4.64                        | 2.64                          | 1.87                            |
| 6       | 44.96   | 28.47                | 2.03                        | 10.43                       | 5.54                          | 4.55                            |
| 7       | 53.02   | 118.40               | 1.80                        | 8.75                        | 7.06                          | 1.96                            |
| 8       | 58.95   | 33.12                | 2.57                        | 7.31                        | 6.13                          | 1.27                            |
| Dog No. | INSR  | CEACAM1 | IDE  | INSR  | Albumin (g/dL) | Total Bilirubin (mg/dL) | AST (IU/L) | ALT (IU/L) |
|--------|-------|---------|------|-------|---------------|------------------------|-----------|-----------|
| 9      | 66.05 | 27.27   | 2.54 | 9.80  | 7.51          | 2.40                   |           |           |
| 10     | 71.92 | 32.96   | 2.46 | 8.33  | 6.73          | 2.15                   |           |           |
| 11     | 72.05 | 23.27   | 1.96 | 26.23 | 18.0          | 8.69                   |           |           |
| 12     | 77.17 | 32.45   | 2.0  | 10.44 | 8.50          | 2.16                   |           |           |
| Mean ± SEM | 50.36 ± 5.51 | 48.08 ± 8.93 | 2.03 ± 0.12 | 9.37 ± 1.67 | 6.69 ± 1.18 | 2.70 ± 0.60 |

FPE, first-pass hepatic insulin extraction; SI, insulin sensitivity; SiP, peripheral insulin sensitivity and SiHGO, hepatic insulin sensitivity.

Table 2. INSR, CEACAM1 and IDE relative gene expressions; fasting plasma levels of albumin, total bilirubin, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) of all animals.
|   |      |      |      |      |      |      |  |  |
|---|------|------|------|------|------|------|---|---|
| 2 | 1.77 | 0.21 | 0.24 | 1.77 | 3.6  | 0.2  | 20 | 30|
| 3 | 0.03 | 1.39 | 3.05 | 0.03 | 3.1  | 0.1  | 15 | 17|
| 4 | 4.54 | 0.45 | 1.68 | 4.54 | 2.5  | 0.3  | 23 | 56|
| 5 | 3.31 | 0.40 | 0.46 | 3.31 | 3.4  | 0.2  | 22 | 26|
| 6 | 5.87 | 0.61 | 1.10 | 5.87 | 3.3  | 0.2  | 34 | 40|
| 7 | 4.14 | 3.49 | 0.22 | 4.14 | 3.2  | 0.2  | 33 | 36|
| 8 | 2.35 | 0.92 | 0.32 | 2.35 | 3.2  | 0.2  | 28 | 27|
| 9 | 3.45 | 0.21 | 0.19 | 3.45 | 3.3  | 0.2  | 28 | 41|
|10 | 3.33 | 2.01 | 2.16 | 3.33 | 2.7  | 0.1  | 20 | 24|
|11 | 5.2  | 2.46 | 5.17 | 5.2  | 3.3  | 0.1  | 33 | 52|
|12 | 7.72 | 1.08 | 8.58 | 7.72 | 3.4  | 0.1  | 23 | 41|

INSR expression relative to 18s rRNA, CEACAM1 expression relative to 18s rRNA, IDE expression relative to 18s rRNA, fasting plasma albumin, total bilirubin, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels of respective dogs.

**Figure legends**

Figure 1. Paired portal/peripheral insulin infusion method for measuring first-pass hepatic insulin extraction (FPE).

(A) Insulin profile during the portal/peripheral insulin infusion experiments. Insulin 1; 3.0 and 1.5 pmol/kg/min intraportal and peripheral infusions respectively. Insulin 2; 6.0 pmol/kg/min intraportal and 3.0pmol/kg/min peripheral infusions. Insulin 3; 9.0 and 4.5pmol/kg/min intraportal and peripheral infusions respectively.
(B) Infusion rate vs. its steady state plasma insulin, with slope, m. For portal infusion, correlation coefficient, r = 0.98 and 0.99 for peripheral infusion. Slope of the portal infusion plot, m_po, was 25.8 kg*min/ml and that of the peripheral infusion, m_pe was 52.0 kg*min/ml for n = 12. FPE = [1-(m_po/m_pe)] = 50.4%, for derivation of equation refer to calculations under methods.

Figure 2. Correlations between FPE, insulin sensitivity and fasting insulin.

Correlations between (A) FPE vs. insulin sensitivity (SI); (B) FPE vs. fasting insulin; and (C) SI vs. fasting insulin. (A) FPE vs. SI; rs = 0.74, p = 0.006, (B) FPE vs. fasting insulin; rs = -0.64, p = 0.03 and (C) SI vs. fasting insulin; rs = - 0.67, p = 0.02

Figure 3. Correlations between FPE vs. SiP and FPE vs. SiHGO

Correlation between (A) FPE vs. SiP (r_s = 0.80, p = 0.002); and (B) FPE vs. SiHGO (r_s = 0.31, p = 0.33).

Figure 4. Correlations between FPE vs. INSR; FPE vs. CEACAM1 and FPE vs. IDE

Correlation between (A) FPE vs. INSR (rs = 0.67, p = 0.02); (B) FPE vs. CEACAM1 (rs = 0.43, p = 0.16); and (C) FPE vs. IDE (rs = 0.31, p = 0.33).
Figure 5. Correlations between Fasting HGO vs. INSR and Fasting HGO vs. FPE

Correlation between (A) Fasting HGO vs. INSR ($rs = 0.21$, $p = 0.51$) and (B) Fasting HGO vs. FPE ($rs = 0.50$, $p = 0.10$).

Figure 6. Distribution of FPE.

Distribution of FPE of 18 dogs. Mean of 53.4%, indicated by the broken line and SD of 19.5%. Baseline data of 6 dogs, already published (3) were included to increase the sample size in the assessment of the FPE distribution.
Figure 1
Figure 2
Figure 3
Figure 4

A. INSR (arbitrary units) vs. FPE (%)

- $r_s = 0.67$
- $p = 0.02$

B. CEACAM1 (arbitrary units) vs. FPE (%)

- $r_s = 0.47$
- $p = 0.12$

C. IDE (arbitrary units) vs. FPE (%)

- $r_s = 0.31$
- $p = 0.33$
Figure 5
Individual dogs

FPE (%)

Figure 6